

The end of the Baltimore saga

One of the most corrosive disputes of recent years in the research community should be ended with the open acknowledgment of the error of an excess trust by the principal in the case.

DR David Baltimore, president of Rockefeller University in New York, has done the decent thing. In his statement on page 94, he acknowledges that he was wrong to have defended the data of his colleague Dr Thereza Imanishi-Kari in the face of mounting doubts. He has also implicitly acknowledged that his truculence before the Dingell Committee two years ago was, to say the least of it, inappropriate; now he says that government has a legitimate right to inquire into the uses made of public money in research. Above all, he offers an apology to Dr Margot O'Toole, the postdoctoral fellow in Imanishi-Kari's laboratory who first became suspicious of the data published in 1986. That will not erase her painful experiences, but it is a handsome apology.

So what happens now? Has Baltimore said enough to bring an end to the process of inquiry that has consumed the past four years of many people's lives? There is some unfinished business, but one thing is clear: Baltimore has said enough to restore his own reputation as a fine scientist, a man of public spirit and a potentially superb and certainly imaginative president of an outstanding and distinctive research university. Some among his colleagues may be tempted to use this public acknowledgement of error by their leader as an excuse for furthering their own narrow causes, but they should instead reflect on what Baltimore's ingenuity may eventually accomplish for their institution. To make an error may reflect on a person's judgement, but to confess it in the circumstances in which Baltimore now finds himself is a mark of courage. He deserves a break.

Opportunity

Others will be less fortunate. The National Institutes of Health (NIH) have yet to say what will happen to Imanishi-Kari, against whom the charge of fabricating data had been laid. She is an able scientist, if a poor record keeper. What the Office of Scientific Integrity demonstrated last month is that data in a notebook submitted to the Dingell Committee had been cobbled together from different and irrelevant sources, but the status of the originally published data is still not clear. In one sense, it no longer matters, for the article concerned has been withdrawn. But both NIH and the Dingell Committee will want to know how this state of affairs arose. It is especially important that Imanishi-Kari should be given an opportunity to put her case. It is unlikely that this case would have been so prominent had it not been for the association with Baltimore.

The question will also arise of how an article now acknowl-

edged to be unsound could have been published in a journal holding to rigorous standards of peer review. Baltimore himself says that his defence of his co-author was sustained by his "trust in the efficacy of the peer review process". But reviewers cannot tell whether data in an article submitted for publication are false except when there are internal inconsistencies or flagrant discrepancies with data already published elsewhere. Reviewers, rather, judge whether conclusions follow legitimately from the data offered in their support. In the disputed article, suggestive of an unexpected interaction between endogenous and transplanted genes in a mouse, the conclusions did not ring out with the clarity of a bell. The reviewers might well have asked that the work should have been confirmed before publication — and would probably have been scorned. It is therefore to be hoped that one outcome of this case will be a more proper recognition (especially in the Congress) of the limitations of peer review.

Pitfalls

So how can fraud be avoided in the future? Baltimore promises to "participate actively" in working out new guidelines for dealing with allegations of fraud and for protecting those who make them. Fine. But what can be done? This and other cases point to the importance of full and contemporaneous laboratory notebooks. More important still, they demonstrate the pitfalls for institutions that embark on investigations in the belief that allegations of misconduct are almost by definition unfounded. Whistleblowers have a lonely and thankless task which they are unlikely to take on lightly. Institutions have a duty to be more diligent than has usually been the case. They will thus safeguard not merely their own reputations, but also their independence. The mere existence of the Office of Scientific Integrity is a mark of how much they have lost already.

The incidence of misconduct also needs attention. By now there is ample evidence that the driving force lies in the competitive character of academic research, especially in the United States. People cut corners or even fabricate data so as to simulate success and earn the rewards it brings — another grant, perhaps, or even a promotion. The same competitiveness has also made US biomedical research into the most successful scientific enterprise anywhere. The problem is how best to abate the evil consequences of competition without losing the benefits. That, more usefully than the development of further guidelines for the conduct of investigations, is the issue into which Baltimore should now put his energies. □



Market heats up for US biotech companies

- Record amounts of capital raised
- New products pave the way

Washington

AFTER four years of uninspired performance, biotechnology stocks have once again become hot properties on Wall Street. Since the beginning of the year, 20 biotechnology companies have raised a staggering \$915 million of new capital in the public markets — a figure that already exceeds the amount raised during the whole of 1986, when the last wave of financial offerings flowed out from the industry.

Financial analysts say that the renewed interest in biotechnology stocks stems from several factors. As more products are introduced into the marketplace and as companies begin to make meaningful profits, investors are beginning to view the technology as having less risk. In particular, the commercial success of erythropoietin (EPO) has shown that genetically engineered drugs have the potential to be quite lucrative. And a couple of favourable patent decisions have reassured investors that companies will be able to reap the profits of successful invention.

The upshot is that the biotechnology industry is "at the right place at the right time", says Linda Miller, stock analyst with Paine Webber.

The lion's share of the money raised through public stock offerings has been snapped up by top-tier, well established companies, but six little-known firms have amassed more than \$200 million by going public. Almost half of this amount — \$99 million — was raised by Regeneron Pharmaceuticals Inc., a neuroscience company established only in 1988.

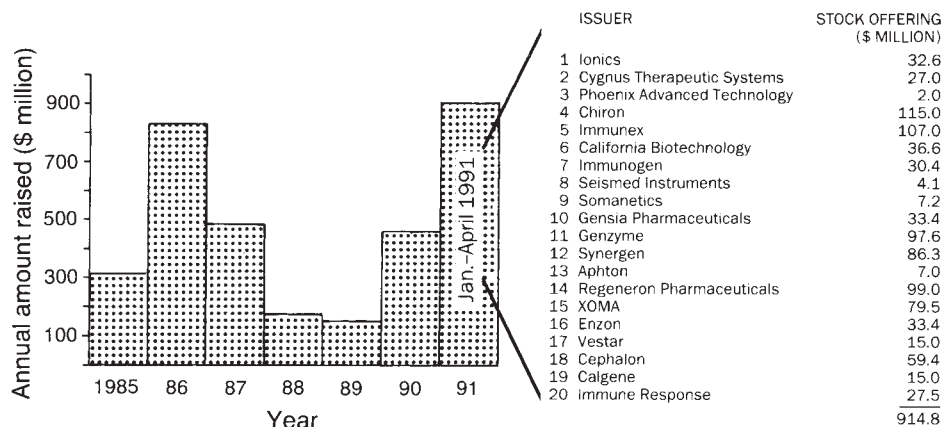
Regeneron's research focus is the development of biotechnology-based compounds for the treatment of neurological disorders, such as Alzheimer's disease and Parkinson's disease. What makes the company's ability to raise \$99 million so striking, says John Gorton, a stock analyst with Van Kasper, is that "the development and commercialization of any products is years away".

The proceeds from that stock offering, which are mostly earmarked for research and development, will enable Regeneron to maintain its current and planned operations well into 1994.

The success of Regeneron and other biotech companies in raising capital demonstrates a renewed faith on the part of investors in the potential of fundamental biotechnology research. Early enthusiasm for biotechnology products on Wall Street was dampened when tissue plasminogen

activator (TPA), hailed as biotechnology's flagship product, fell short of sales targets. Data from two comparative trials of clot-busting agents indicated that TPA is no more effective than streptokinase, a drug produced by more conventional means at one-tenth of the price.

Interest revived last year, however, when EPO, another biotech drug, had worldwide sales of \$630 million. The drug, which stimulates the body to produce more of its own red blood cells, is used to treat anaemia in patients on kidney dialysis as well as HIV-infected individuals taking AZT. Sales of EPO are expected to 'mushroom' as other



Source: IDD Information Services, New York

US biotechnology companies have had tremendous success this year in public stock offerings, raising twice as much in the first four months as in all of 1990.

therapeutic uses for EPO are discovered.

More biotech drugs are in the pipeline. Since the beginning of the year, both Amgen Inc. and Immunex Corporation have received US Food and Drug Administration (FDA) approval for their colony stimulating factors; at least eight more biotech drugs should be approved in 1991. After years of anticipation, this steady stream of drug approvals means that more companies will have a source of revenue and will thus be making the transition to profitability.

Adding to the optimism about biotechnology's future are the resolutions of two important patent disputes earlier this year.

Amgen won a resounding and unexpected victory over Genetics Institute for the US patent rights to EPO, and Cetus Corporation successfully fended off a challenge by Du Pont over the polymerase chain reaction. These court decisions should allow investors better to assess the patent risks surrounding a potential biotechnology investment.

Amgen's victory over Genetics Institute in

its fight over patent rights to EPO, which has left Amgen with a guaranteed US market, is one of two factors that has caused the company's stock to more than quadruple in value in the past 12 months. The other is the recent FDA approval of Amgen's second potential blockbuster biotech drug, granulocyte-colony stimulating factor.

The growing interest in biotech companies has pushed executive salaries quite high, according to a recent survey by the management consulting firm of William M. Mercer of Boston. Of the 26 biotechnology companies surveyed, chief executive officers received an average of \$608,300 in total compensation, including stock incentives. This, Mercer says, is 71 per cent more than the average of \$355,300 earned by CEOs of high-tech companies, and 114 per cent more than earned by CEOs in industry as a whole.

"Good biotech executives are hard to find," says Gorton at Van Kasper. Finding a person with the right mix of a knowledge of the industry and an entrepreneurial spirit can make "the difference between success and failure for these companies," he says.

Not every biotech company is having an easy time finding success. Genetics Institute, for instance, has been beset with problems. Not only was it on the losing end in its struggle with Amgen over EPO, but a court decision over US patent rights to TPA has also gone against the company. Now Genetics Institute and its partners are locked out of the US market for both drugs. Just two weeks ago, Gabriel Schmergel, president and chief executive officer of Genetics Institute, announced his intention to withdraw a proposed common stock offering because of "the recent decline in our common stock share price".

Although most analysts believe that the window of opportunity will be open for some time, most agree that only those companies that merit financing will have access to the market. Having had 10 years of experience with the biotechnology industry, investors now have the ability to ask "a lot of tough questions". "Selectivity is still the watchword," Miller says.

Diane Gershon

Ray of hope for the East

Munich

RESEARCHERS in eastern Germany received an important vote of confidence two weeks ago from the Fraunhofer Gesellschaft, a Munich-based organization that supports and directs scientific research at several dozen institutes in western Germany. The Gesellschaft announced that it will create 19 research institutes and outstations in the former East Germany by merging existing groups from a variety of institutions, thus making it more likely that these groups will survive the turmoil brought on by merging science in the east and west.

By pledging DM500 million (about \$281 million) to the 19 institutes, the Gesellschaft, a non-profit research organization with ties to government and industry, is making the biggest single commitment to research in eastern Germany since German unification.

Although it will affect only certain fields of applied research, the Gesellschaft's support is encouraging for researchers who had begun to wonder whether the West would see any value at all in their work.

The decision is also seen as a step towards rebuilding the collapsing industrial base of eastern Germany. The small- and medium-sized companies, known as the *Mittelstand*, which form the backbone of Germany's industrial economy, often depend on having publicly funded research and development activity nearby.

Now, with the Gesellschaft's action, "there will be a higher density of Fraunhofer institutes in eastern Germany than there is in western Germany," says Wilhelm Krull of the science advisory council Wissenschaftsrat, which had a hand in deciding which groups to take over. By setting up a dense network of application-orientated research centres, Krull says, Fraunhofer Gesellschaft is providing a foundation upon which new or revamped industry can build.

Between 1992 and the end of 1994, the Gesellschaft will distribute the DM500 million among the 19 institutions — ten new institutes and nine outstations of existing western institutes. In addition, the Gesellschaft seeks an additional DM70 million to set up even more institutes. The move is expected to save the jobs of at least 950 researchers and supporting staff.

By moving quickly and decisively into eastern Germany, Fraunhofer Gesellschaft has put pressure on the German government to raise its level of commitment to the eastern part of the country. The governments in Bonn and the *Länder* (states) will have to pour in at least another DM300 million just to renovate the dilapidated buildings in which the institutes are housed and make them fit for modern research. Even more money will be required for modern research equipment, especially computers compatible with those used in industry. "We are counting on Bonn and the *Länder* to help."

says Gesellschaft spokesman Alexander Rothhämel.

All 19 institutions will be composed of researchers who worked in East German institutes, universities or industry. Like the general working population, eastern German research workers face an uncertain future now that their organizations, especially the East German Academy of Sciences, are being shut down or reorganized.

Among those fortunate enough to be selected are a number of groups that used to work at the academy, which in the years before unification had been shifting its emphasis increasingly towards applied research. Because of export restrictions on high technology, the academy and East German industry were forced to develop on their own technologies that were widely available in the West. In the process, eastern Germans developed a technological resourcefulness that is impressive to their Western colleagues. The Gesellschaft decision is a concrete indication that they have not been working for naught.

If the new institutes are to survive past their initial three-year period of support by the Gesellschaft, they will have to generate revenue by doing contract research for industry. The Gesellschaft has said it expects the institutes to cover 25 per cent of their costs through contract research. The 38 Fraunhofer Institutes in the west generally cover about 80 per cent of their costs through outside contracts. But the Gesellschaft recognizes that it will take time for the eastern institutes to discover the most profitable fields of research.

The list of groups and institutes to be taken over includes no surprises; among them are the Academy institutes thought to have the best chance of surviving in a united Germany. The significance of the decision by Fraunhofer is that related groups will be allowed to keep working together rather than having to take their chances on the open market. One example is the central institute for cybernetics and information processing in East Berlin, where, in the transition phase before unification, clever programmers had already found western contracts to augment their income. Other fields on the list include solid state physics, ceramics, polymer chemistry and applied optics.

The Gesellschaft left itself two ways out in case the research services that its eastern institutes provide prove to be superfluous. After three years, the Gesellschaft may either close the institutes or move some of the researchers to related western institutes.

Because a move to the west goes against Bonn's philosophy about unifying German research — namely, to keep as much of eastern research as possible in the east — a Gesellschaft spokesman says he expects such a westward shift to occur in only a small number of cases.

Steven Dickman

Auditors fret, NASA persists

Washington

CONTROVERSY over the price of the planned international space station — the one constant in the embattled project's tumultuous decade of design — has struck again. A new US government accounting audit shows that the space station may eventually cost as much as \$40,000 million more than expected. Coupled with an emerging consensus from the scientific community that, as now designed, the space station will be of minimal scientific use, the news may set the stage for the toughest battle yet in Congress over the future of the project.

The audit, by the congressional General Accounting Office (GAO), found that NASA (the National Aeronautics and Space Administration) had not included the costs of such items as a crew-escape module and a artificial-gravity centrifuge in its estimates to Congress. NASA also neglected to include the costs of maintaining and operating the station once it is completed, GAO said.

"Once these costs are added together," GAO comptroller general Charles Bowsher testified at a congressional hearing last week, "what we actually have is at least a \$118 billion program — about \$40 billion to achieve permanent occupancy and about \$78 billion to keep the station operational between 2000 and 2027." In defence of the project, senior NASA officials dismissed the audit findings as a matter of differing accounting methodologies and called for an end to such reviews. "It's time to come to a decision," testified NASA administrator Richard Truly. "Let's give it to the engineers and let them go build it."

Since 1984, NASA has redesigned the space station eight times to adapt to new technical, financial and congressional demands. In the process, the station has shrunk from an eight-person, six-module, 125-kW design whose development was expected to cost \$8,000 million to a four-person, three-module, 56-kW design expected to cost \$16,900 million to develop and \$13,000 million to deploy. In development alone, NASA's expects to pay twice its 1984 estimate for half as much station. And if GAO is right, the real cost may be even higher — \$10,000 million more in the development phase and \$34,000 million over NASA's estimates during the operating phase.

The history of such space station controversies has been consistent. Each new study has revealed that the project will probably cost more and deliver less science than previously promised. Each time, the grim figures have prompted new calls for the project's cancellation. Yet, so far, the US champions of the project have been able successfully to argue that it should still be built.

Christopher Anderson

Award now, pay later

London

UNIVERSITIES that win grants from the UK Science and Engineering Research Council (SERC) may be forced to borrow money to buy equipment and hire research assistants, under a new SERC policy that is designed to relieve the council's immediate financial difficulties.

The reason is that SERC, in its latest round of grants, has imposed strict limits on what percentage of a grant can be spent in its first two years. The details vary from project to project, but some researchers will be given only 15 per cent of a three year grant to spend in the first year. In some cases, SERC intends to hold back more than 50 per cent of a grant until the final year of the project.

This, however, is precisely the opposite of the way that many researchers need their grants to be structured. Grant recipients often need to spend a large proportion of their awards in the first two years of a project, particularly when expensive items of equipment must be purchased. SERC's policy, therefore, will force universities to borrow money to cover the start-up costs of research projects, says David Thomas, industrial liaison officer at Imperial College, London, who is also responsible for Imperial's income from the research councils.

UK RESEARCH FUNDING

Reform may cut grant awards

London

The British research councils would have to cut their grant spending in the universities by as much as 12 per cent if the government goes through with a plan to reform British university research funding, according to a confidential report presented last week to Education and Science Secretary Kenneth Clarke.

From the 1992-93 academic year, the government aims to transfer £100 million a year to the research councils from the research budget of the Universities Funding Council (UFC). The idea is that in the future the research councils will pay the overhead costs (administrative costs, telephone bills and the like) of research projects they support. The universities would then be responsible only for the salaries of permanent academic staff and the costs of running university buildings.

But the new report, prepared by the Committee of Vice-Chancellors and Principals (CVCP) and the research councils, says that £100 million is inadequate to cover the overhead costs of research council-funded projects. The CVCP calculates that £138 million is needed; the research councils put the figure at £149 million. Their analysis is based on the costs of a sample of research projects in ten British universities.

Thomas fears that the policy could "change the criteria by which grants are awarded". Rather than the best projects being funded, he believes grants may go to the richer universities, or those that are prepared to slip into debt. Jim Reed, industrial liaison officer at the University of Surrey, is now conducting a survey of his colleagues around the country to gauge the extent of the difficulties the new policy will bring for the universities.

SERC officials say that the spending limits will help relieve SERC's well publicized cash flow problems (see *Nature* 349, 551; 14 February 1991), but add that there is "no intention to place any additional financial burden on the universities".

The research council is operating under the assumption that its finances will be in a better state two years from now, and that it will then have no problem honouring the grants in full.

SERC chairman Sir Mark Richmond is determined to protect research grant spending from further cuts. But even with the new policy of spending limits for the first two years of new research projects, SERC expects to award only 50 per cent of the usual number of new research grants this year.

Peter Aldhous

If the research councils are to pay the overhead costs of each project they support but are given only an extra £100 million to do so, the report argues, they will have little option but to award fewer research grants.

The proposed transfer has not created a new problem — it has merely made more evident the universities' assertion that they have not been getting paid enough for research for some time. "It is clear that some universities have been paying for the direct costs of projects supported by research council grants to a far greater extent... than has been recognized," the CVCP concludes in the report. And Derek Roberts, provost of University College London, argues that the underfunding of research costs in the universities is some £100 million more than suggested by the research councils: the report excluded the cost of mainframe computing, and did not consider projects funded by charities.

The report, which was commissioned by the government, should put pressure on the Department of Education and Science (DES) to provide more money for research. But Clarke is not expected to abandon the planned transfer, which has already been delayed by one year. The DES is convinced that the transfer will improve the administration of research spending.

Peter Aldhous

Mining ban in the air

London

MINING in Antarctica would be banned for at least 50 years if the governments of the Antarctic Treaty nations sign a draft protocol on Antarctic environmental protection produced last week at a meeting in Madrid. But it is not clear whether the Bush Administration in the United States will approve the version of the treaty agreed to by its negotiators, and a rejection of the draft could yet prevent consensus on the divisive mining issue when Antarctic Treaty nations meet next month.

The draft text would ban mining and oil exploration for the next half a century. Even after then, the ban could be relaxed only if all of the present 26 consultative parties to the Antarctic Treaty agree — generally seen as an unlikely prospect.

The protocol has pleased environmentalist groups and those governments, led by Australia and France, that were pushing for a permanent ban. James Martin Jones, from the World Wide Fund for Nature, says agreement on the draft "would give us what could be described as an indefinite ban."

Before the meeting, a group of nations led by Britain and the United States was expected to ensure that the option of mining in the future was kept open. But the group was weakened by a surprise policy reversal by Japan, which was previously a strong supporter of the mining option (see *Nature* 348, 570; 1990). The Japanese delegation announced at the start of the meeting that Japan would support a ban that could be lifted only by consensus.

Observers in Madrid now expect the United States to be the only possible obstacle to agreement on the draft protocol. The draft is understood to have gone well beyond the negotiating brief given to Curtis Bohlen, head of the US State Department delegation.

The US administration has until a second meeting in Madrid, beginning on 17 June, to consider the draft. State Department officials last week refused to comment.

Britain, however, is expected to accept the draft. The UK Foreign Office has said that consensus on the mining issue is its number one priority, and rejection of a draft supported by the vast majority of Antarctic nations would be politically embarrassing.

Aside from the mining ban, the draft environmental protocol contains new provisions to assess the environmental impact of proposed research projects in Antarctica. Antarctic scientists had feared that a new regime of environmental impact assessment would place undue restrictions on Antarctic research. Under the new draft, the environmental impact of some research projects will have to be assessed. But this will be carried out by national authorities, and is not expected to place obstacles in the way of research.

Peter Aldhous

ENDANGERED SPECIES

Emerging virus threat

Washington

IN January, a small group of golden lion tamarins waited in a holding facility at the National Zoological Park for a trip to Brazil. Eleven of the foot-long monkeys were to be released into the wild as part of the zoo's reintroduction campaign for endangered species. Then, three days before the tamarins were scheduled to leave, zoo pathologists discovered that one of the monkeys posed a potential threat to South American wildlife. The tamarin was carrying antibodies against the callitrichid hepatitis virus (CHV), an infectious organism that has recently struck primate populations in nearly a dozen US zoos. Indigenous to Old World primates, the virus has never been seen in the New World outside captivity.

The pathologists, who already knew the tamarin to have been exposed to CHV, learned at the last minute that the virus could be transmitted to other animals through rodents — a discovery that made it much more threatening. If the monkey had carried CHV into the wilds of Brazil, where the virus has never been seen, it might have spread to other primates and other susceptible species.

The consequences would be impossible to predict. The virus might have died out with no effect. But the pathologists had to consider the worst-case scenario — that the virus might have caused a plague that would devastate some vulnerable species.

The experience with the tamarins highlights a dilemma facing reintroduction programmes. Although the value of importing endangered species back into their natural habitats is unquestioned, some researchers are now beginning to reexamine the safety of such campaigns.

The danger arises from what are known as 'emerging viruses'. Like AIDS and hepatitis, emerging viruses are previously non-threatening viruses that can decimate new populations by acquiring fresh hosts and vectors — often with the unknowing help of humans who introduce new species into virgin environments. Canine distemper, for example, has wiped out the black-footed ferret in the wild and was most probably introduced by domestic dogs.

Reintroducing an existing species into Does this golden lion tamarin carry an 'emerging its own original environment can also risk virus'?

an epidemic, especially when those animals have been exposed to the cocktail of viruses that proliferates in a zoo. In the case of the hooded crane, a probably foreign herpes virus has slowed reintroduction and further endangered the last of the surviving wild population.

"It's a very serious potential problem," says Benjamin Beck, associate director for biological programmes at the National Zoo. "It's only because of our advanced diagnostic facilities that we were able to catch this virus. Who knows what else is going

through?" In response, some countries have essentially prohibited reintroduction by instituting such strict import regulations that even animals only recently removed from the wild cannot be returned.

Yet most researchers believe that they have little choice but to go ahead, albeit cautiously. Devra Kleinman, the National Zoo's assistant director for research, says, "To maintain maximum genetic diversity we're going to have to inject animals into the wild. Plague is a risk, but it's a risk we feel we have to take."

Six other US zoo programmes are participating in reintroduction and breeding projects for tamarins, among some 55 other endangered species that are now being reintroduced. To minimize the danger of an epidemic, zoo staff quarantine tamarins for several months before reintroduction, during which time the animals are examined for parasite eggs, viruses and genetic problems. "We check for the things we know," says zoo pathologist Richard Montali. "Anything that we can treat, we treat. Any animals we have questions about, don't go." Overall, about 15 per cent of the tamarins are disqualified for one reason or another.

After the animals are released in Brazil, seven zoo specialists observe them every day over a six-month quarantine period in a remote area away from wild tamarins. If the researchers spot signs of a disease outbreak, they can trap and remove the monkeys within hours.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

National Zoological Park

foreign pathogens and parasites. Some of the viruses affect the animals themselves, others may simply use them as a carrier.

In the case of CHV, pathologists believe that rodents carried the organism from Old World to New World primates at a zoo, probably through the baby mice (known as 'pinkies') with which the tamarins are fed. Another tamarin disease, caused by a parasite found in bush babies (small simians that are also kept at the National Zoo) appears to use insects as its vector. Cockroaches, which are as common in the tamarins' zoo quarters as they are in nearby Washington apartments, are a favourite tamarin snack and the chief suspect in that case.

"The diseases are evolving faster than we are identifying them," Kleinman says. "The parasites are constantly changing to keep one step ahead of the hosts."

Such a moving target makes complete screening impossible in reintroduction programmes. New viruses are going to get through, if they have not already. Zoo officials hope that they can screen out the worst of the risks, but they admit that they are gambling on good luck and quarantines.

"When we decided in the beginning to go ahead with the reintroductions, we didn't know exactly what the risks were, and we still don't," Kleinman says.

The researchers point out that other human activities, from agriculture to ecotourism, are also bringing new diseases to wild populations. "We're exposing Brazil to less risk by exposing it to our tamarins than we are by exposing it to ourselves," Beck argues.

While that may be true for the country at large, something like an infected tamarin can be the ultimate nightmare for the species that the researchers are trying to save. Unlike the zoo-bred animals, the wild populations have not had generations in which to become immune to the foreign virus.

Most researchers agree that the best solution in many cases would be *in situ* conservation — the breeding and management of endangered species within their natural environment. If the animals can be protected in areas near where they are naturally found, the risk of their being exposed to new diseases is minimized.

But conservation programmes are expensive, and most are funded by zoos, which in turn depend on public support. Although reintroduction programmes may be intellectually attractive, people are unlikely to support the effort if they cannot see the animals.

So most zoos, strapped for cash, continue to run their reintroduction programmes in the high visibility of captivity. They screen for what diseases they can, and hope for the best with what they miss. "It's an area where we're playing God," says Michael Hutchens, director for conservation and science for the American Association of Zoological Parks and Aqauria. "But we don't have a lot of choice."

Christopher Anderson

MITI to shake up institutes

Tokyo

THE first major reorganization of Japanese government laboratories in decades is now being planned by the Agency of Industrial Science and Technology (AIST), an arm of the powerful Ministry of International Trade and Industry (MITI). The plan to shuffle several research institutes in Tsukuba science city is intended to inject new life and greater flexibility into the government research system.

Tsukuba science city, which was created in the 1970s by uprooting government research institutes from Tokyo and transplanting them to the rice paddy fields of Ibaraki Prefecture north of the capital city, has come to life in the past few years with a sudden influx of private sector research institutes (see *Nature* 345, 378; 1990). But while some government research laboratories are flourishing in the new environment, others are bogged down in bureaucracy and an organizational structure that dates back to the beginning of this century. The latter category includes the four AIST institutes targeted for reorganization, which one leading optoelectronics researcher in Tsukuba calls "dinosaurs".

Those institutes are the National Chemical Laboratory for Industry, the Fermentation Research Institute, the Research Institute for Polymers and Textiles and the Industrial Products Research Institute. They have a total of more than 500 researchers and a combined annual budget of over ¥8,000 million (\$60 million).

If the AIST plan is put into effect, the four will be combined into two new institutes, one specializing in life sciences and the other in materials science.

In addition, AIST will establish an entirely new research centre for interdisciplinary research that will focus on nanotechnology and 'holistic science'.

Researchers in the life sciences and materials science are now scattered among various divisions of the four AIST institutes that are huddled together in one section of Tsukuba. The current plan is to bring all the life scientists into the present Fermentation

Research Institute and a new building to be constructed nearby, while putting the material scientists in the National Chemical Laboratory and the adjacent Research Institute of Polymers and Textiles, which will also get a new wing. The Industrial Products Research Institute, meanwhile, will be converted into the new interdisciplinary research centre, although a section of it will be set aside for the life scientists.

AIST officials expect the total outlay for new construction work to amount to ¥4,500 million (\$33 million) over the next three years. The agency has already allocated ¥410 million (\$3 million) for this fiscal year with which to begin construction of the new building.

The most radical element of the plan is the proposed new interdisciplinary research centre, which has several aspects that depart from normal Japanese practice.

Instead of having permanent staff employed on a lifetime basis — as is now the case in all government research laboratories — researchers will be assigned to the centre to work on projects for periods of three to five years. At the end of that time, the projects will undergo "severe" review, says Masayoshi Hamada of AIST, who is in charge of the reorganization.

Researchers will be drawn not only from AIST laboratories but also from private industry, universities and overseas. Non-Japanese scientists will be involved in the planning stages of the projects, rather than just being invited as 'guests'. Hamada expects the centre to have a total complement of about 100 researchers.

In many respects, the new centre will resemble the Research Center for Advanced Science and Technology (RCAST) of Tokyo University and the Frontier Research Program (FRP) of the Institute of Physical and Chemical Research (RIKEN), based in the outskirts of Tokyo. RCAST and FRP are two successful anomalies recently created in the government research sector. RCAST has a number of chairs funded by private industry. RIKEN is a 'special corporation' (*toku-*

Thier plans to leave Institute of Medicine

Washington

SAMUEL Thier, who presided over the US Institute of Medicine (IOM) for six years of unprecedented growth, is leaving to become president of Brandeis University. He said last week that he plans to leave IOM by 1 October, and that the institute is in the process of assembling a search committee to find a new president.

Part of the National Academy of Sciences, IOM is an independent body of scientists who provide the federal government, Congress and others with advice on biomedical issues. During Thier's tenure, the amount of IOM contract studies grew from some \$3 million to \$14 million, and it now has nearly 30 studies under way. In addition, an endowment was created and grew to the current level of \$20 million.

Asked why he is leaving for academic life at a time when IOM is doing well and the university community is facing cutbacks and stalled growth, Thier said that he is "intrigued by challenge". Brandeis, as the smallest and the youngest of the members of the research orientated Association of American Universities, "is an opportunity to do some interesting experiments in reorganization", he said.

Before joining IOM in 1985, Thier was chairman of the department of internal medicine at the Yale University School of Medicine. Christopher Anderson

shuhoin) and, although it is affiliated to the Science and Technology Agency, it has much greater freedom and flexibility to establish programmes such as FRP than do ordinary government research laboratories.

The AIST centre will concentrate on two seemingly contradictory themes. One group of researchers working in an 'atom factory' will examine and manipulate biological and inorganic material at the level of atoms and molecules with scanning tunnelling microscopes, atomic force microscopes and other devices for working at the nanometre level. A second group, working in the opposite direction from this reductionist approach, will study whole organisms, societies and technologies holistically.

AIST still faces one hurdle before its new plan can be implemented. The reorganization has to be approved by the Management and Coordination Agency, which, under a policy of fiscal restraint implemented in the early 1980s, is steadily trimming the number of government employees, including researchers, to try to cope with the national government's chronic debt. AIST will submit a formal proposal to the agency in late August and a decision will be made at the end of the year. But AIST officials say they are confident of success because their plan does not require any increase in the number of permanent government employees.

David Swinbanks

Moving to nanotechnology

Tokyo

AIST's decision to establish an 'atom factory' in a new research centre in Tsukuba (see above) is part of a massive move by MITI to promote research into nanotechnology.

This fiscal year, MITI will launch a 'next generation' (*jiseidai*) project to develop quantum dot and quantum well devices, with a budget of about ¥5,000 million (\$40 million) over ten years. Several major electronics manufacturers are expected to join the project this summer (see *Nature* 349, 449; 1991).

And the *jiseidai* project is only the beginning. MITI officials are now sketching out a large-scale project that, with the collaboration of dozens of companies, will plough hundreds of millions of dollars into the development of 'ångström technology'.

This proposal is a strong candidate for inclusion in MITI's budget request for fiscal year 1992, to be submitted at the end of August. The eventual intention, says Masayoshi Hamada of AIST, is to link the proposed atom factory in Tsukuba with the ångström technology project. D.S.

Turmoil in European biology

Lennart Philipson

A power struggle between various European organizations is hindering attempts to expand basic biological research in Europe. A remedy to this situation must involve both scientists and top politicians.

AMONG the natural sciences, biology and especially molecular biology, is growing most rapidly and shows great promise for the future. Biotechnology, fed by the discoveries of basic molecular biology, is rapidly gaining momentum. Molecular biology also appears to be a necessary ingredient in medicine, agriculture and attempts to correct major faults already introduced into the environment, such as overfertilization of the soil, contamination of air and water and exhaustion of energy resources.

The Human Genome Project and related genome projects, together with the approaching economic unification of Europe, have led to an increase in proposals for new projects in biology to be organized and supported at the European level. These same proposals have, unfortunately, also revealed the animosity and struggle for power within and between the different European organizations involved in funding biological research. It has become abundantly clear that we need — but do not have — a coordinated European policy for the support of basic and applied research in biology.

The players

The players in the field are numerous. The organization with the most substantial funds for European collaborative projects is the European Commission (EC). It concentrates foremost on applied projects directed from the top which are often initiated without proper consultation with the scientific community. Through the single European Act, the EC has its own revenue which is constantly growing, mainly as a result of the reduction in the subsidies of agricultural products. At the political level, EC proposals are subject to review and approval by the European Parliament and the Conference of Science Ministers.

The European Science Foundation (ESF) depends mostly on the resources provided by the research councils which are its constituent organizations. This inevitably leads to a conflict between the truly European and the national interest. Research council money provided to the ESF cannot be spent nationally, with the result that the ESF's budget is kept at a minimum level.

The European Molecular Biology Organization (EMBO) and the European Molecular Biology Laboratory (EMBL), both devoted to basic research in molecular biology, receive their funds directly from the 17 and 15 member states respectively. The national delegates are at a much lower political level than those at the EC and, in one mem-

ber state, the funds for the European Molecular Biology Conference (EMBC) and EMBL are in competition with resources for national projects.

HUGO (the Human Genome Organization), a global organization aimed specifically at promoting the Human Genome Project, has so far only support from private foundations which, of course, leads to a weaker position in the political arena.

EMBO and the EMBL have for several years pointed out, in vain, the need for a stronger European base in basic molecular biology. They have put forward proposals which involve requests for additional money for more pre- and postdoctoral fellowships, for a programme of research groups and even for additional EMBLs. This package of proposals was strongly supported by a majority of the EMBO membership. A necessary expansion of the EMBL DNA Data Library into an independent European Bioinformatics Institute was not included in that package, but it has received support from an ESF report on the Human Genome Project and from an EC-funded report from the European chemical industry.

Several European science societies are also competing for these same funds. The European developmental biologists in the European Developmental Biology Organization (EDBO), the ecologists in the European Environmental Research Organization (EERO), the plant molecular biologists, the cell biologists in the European Cell Biology Organization (ECBO), the neurobiologists and the structural biologists are all separately making attempts to secure funds from the EC and the ESF, for fellowships, training programmes and workshops in their specialist fields. The biochemists have unified their national organizations in the Federation of European Biochemical Societies (FEBS) which, using revenue from FEBS journals, arrange fellowships and workshops. (See table for list of acronyms used in this article.)

The struggle

The power struggle was spotlighted when the EC stopped payments of grant support already contracted for under the SCIENCE programme, which has been making grants for basic research carried out in collaborations. These breaches of contract were precipitated by a commissioner who failed to receive support for his grandiose EC fellowship programme from the European Parliament and the Conference of Science Ministers. Another conflict arose when the German science minister, probably in need

of arguments that would convince the national green politicians of the good sense of the genome projects, simultaneously requested reports on human genome research in Europe from the ESF and the Academia Europaea.

These reports presented conflicting views on the future coordination and organization of the programme. The academy suggested an EMBO-like organization, 'Eurogene', for the coordination of the national programmes. ESF, on the other hand, favoured the view that the genome projects should mainly be national enterprises coordinated to avoid unnecessary duplication.

These conflicting views reflect the constitutions of these sources. The Academia Europaea, a European organization consisting of scientists, naturally proposes a pan-European activity, but a foundation deriving most of its support from the national research councils is more inclined to propose networking of national projects. From all the reviews and reports on expanded European projects in biology it is clear that the EC, which is the only organization with its own European funds, favours European projects, whereas the ESF, an organization with funds derived from national sources, tends to defend the national interest.

It is interesting that the majority of those who have been engaged in these numerous reviews are members of the EMBO, and so should have a natural channel for advocating European support through their government representatives on the EMBC, which finances the EMBO. Many of the proposed European projects in biology could be managed by the EMBO if it had a chance to expand. The EMBO members, some 750 of the top biologists in Europe, constitute a coherent group that should be able to work out the

ACRONYMS USED IN THIS ARTICLE

EC	European Commission
ECBO	European Cell Biology Organization
EDBO	European Developmental Biology Organization
EERO	European Environmental Research Organization
EMBC	European Molecular Biology Conference
EMBL	European Molecular Biology Laboratory
EMBO	European Molecular Biology Organization
ESF	European Science Foundation
FEBS	Federation of European Biochemical Societies
HUGO	Human Genome Organization

priorities among themselves and present their proposals more strongly to the EMBC. European biologists are, however, obviously keener to seek support for their specialist area from whatever source looks promising than to remain united and show loyalty to their own organization. It no doubt also suits the EC to offer small sums of money here and there; to divide is to rule.

The problem

Why does Europe have all these competing organizations and why do we lack a body that can harmonize the programmes presented by the EC, ESF, HUGO, EMBL and EMBO, not to mention ECBO, EDBO, EERO and the other European subject-oriented interest groups?

There are several reasons for this competition and the polarization it engenders. Interestingly, most of them lie outside the most obvious cause — the limits on research funds. In most European countries, biological research is well supported even if many research councils have difficulties in executing objective peer review or in taking unpopular decisions resulting from the changes in the quality of research or of national priorities. The genome projects, which rapidly received political support, gave the national research councils in many countries extra funds. One conclusion from the response to the Human Genome Project is that scientists in unison can easily convince politicians to provide additional support so long as they present coherent, understandable and attractive proposals. Another factor, of course, is that European politicians, frightened by the US lead in the Human Genome Project, supported European efforts from fear of being left behind.

A second reason for the failure to coordinate European research programmes may be the large group of science administrators who influence decisions at both national and international levels. Most of them have either failed in research or have been trained in law or economics. Even when they have some insight into current scientific problems, their loyalties rest entirely with the organization or the ministry for which they work; they strive to provide identity and importance to their administrative unit rather than to find real solutions to scientific organizational problems. In the rapidly growing field of biological research, where we have difficulty in training sufficient numbers of scientists both for university and industry, this gulf between insufficiently and inappropriately trained administrators and the research community may be greater than in the field of physics, where 30–40 years ago several highly trained physicists opted for administrative rather than scientific careers.

A third contributing factor is certainly that the organization which has by far the largest funds for European collaborative projects, the EC, has a questionable peer review process and often distributes funds on geographical rather than scientific criteria. The

One reason for the failure to coordinate European Research programmes may be the large group of science administrators who influence decisions at both national and international levels. Most of them have either failed in research or have been trained in law or economics . . . their loyalties rest entirely with the organization or the ministry for which they work . . .

limited call mechanism, which restricts applications for support to selected scientists chosen by dubious criteria, is clearly detrimental to a sound research activity.

Finally, many representatives of the national science research councils favour the idea that basic science should not be Europeanized, but instead, that national efforts should be maintained and strengthened. As well as national pride and the desire to maintain expertise on a national basis, the chief political reason for this policy is that it provides the basis of growth for national industry. But that is not really true. All the relevant industry in this field is multinational, and does not rely on the science effort of single countries. Industry in fact has often more information about relevant partners than the scientists themselves. For instance, the industrial partners Amersham and Bertin have already licensed the multiple oligonucleotide synthesizer developed at the EMBL to be used in the French-British Eureka project on sequence automation.

From the scientific side, increased Europeanization offers great advantages. Science can be measured only by international standards. Peer review on a European basis can be performed in a more objective, independent and professional way. (This is especially important for the small countries of Europe.) The skewed distribution of graduate and postdoctoral students can be evened out. It is not feasible for each country in Europe to build up in every field the expertise, which is now the entrance ticket for access to the ever increasing flow of not-yet-published information. Scientific breakthroughs are unpredictable but are fostered by an open research environment containing scientists with different scientific and cultural background, as is clearly demonstrated in the United States.

The remedy

How can we then ease the ever increasing polarization and competition between the different European organizations?

First the scientists themselves must demonstrate that they can set the priorities, that they are prepared to make themselves available for peer review on an international scale and that they can recognize good science within their own subject area but outside their own country. We cannot leave it to the science administrators to achieve the unification because their first loyalty goes to their

employer. EMBO has a long record of providing international peer review and given the authority to launch an expansion of European collaboration in several areas of molecular biology it could easily draw on its qualified membership to set up planning committees for such programmes. The plan could then be circulated to all national or European bodies for comments and amendments. This science-based scheme could well become as successful as the current fellowship and course committees of the EMBO and, in the past, the EMBO committee involved in harmonizing the recombinant DNA-technology guidelines. In the 1970s the latter set an example to national legislators of how to separate facts from fiction.

There is also a need for a joint coordinating body for all the various organizations representing both basic and applied areas of biological research in Europe, especially now as we attempt to unify the funding of research beyond the borders of western Europe. It cannot be desirable that organizations representing the research community in basic biological science lack direct contact with the highest levels in the national ministries and as a consequence receive less support than projects in the applied sectors, which are not infrequently inefficiently supervised by bureaucrats and politicians.

A yearly pan-European Conference of Science Ministers should be established to be responsible for coordinating European projects and overseeing and supporting most established European organizations representing basic or applied science. Some diversity may be beneficial. The proposal to fuse all European organizations under the EC, as has been suggested, has little to recommend it, because that would place the scientists under the tutelage of unqualified administrators. The EC furthermore suffers from an opaque, bureaucratic, time-consuming, costly and laborious administration that cannot be fountain-head of a European science programme. Proposals from the EC can at present only be vetoed or approved by the Conference of Science Ministers but the latter has no real coordinating power. A pan-European conference of science ministers that can provide the resources directly to the different European organizations based on competing proposals would assure a direct involvement of elected politicians in the harmonization of the European science programmes. Each country may in these deliberations support or reject individual proposals and pay relatively to its gross national product in a similar manner to that successfully applied in the industrial Eureka projects. The administrative costs could be kept at a minimum with this system because it places the management of the funds in the hands of the scientists. □

Lennart Philipson is at the European Molecular Biology Laboratory, Postfach 10.2209, Meyerhofstrasse 1, 6900 Heidelberg, Germany.

Silver Spring monkeys

SIR — As one who attended both State of Maryland vs. Edward Taub trials and served on the search and seizure warrant team that removed documents and animals from Taub's laboratory, I wish to comment on S. B. Galsworthy's letter (*Nature* 349, 13; 1991).

Galsworthy writes that "Taub was never convicted". Indeed, he was — by a circuit court judge and then, in a later trial *de novo*, by a district court jury. His convictions were for violations of Article 27, Section 59 *et seq.* of the Annotated Code of the State of Maryland, otherwise referred to by law enforcement personnel as "the anti-cruelty statute".

I saw inside Taub's laboratory. The place was abominably filthy, its rusted cages with their broken bars failed to meet federal minimum standards (a matter of public record), all medications had long expired (as shown in the police photographs admitted into evidence), and animals languished with infected wounds (as confirmed in the testimony of primatologist Dr Geza Teleki and four veterinarians with expertise in primate medicine who personally examined the Silver Spring monkeys).

Taub's conduct should be used as an example of how not to do things in the laboratory.

INGRID E. NEWKIRK

People for the Ethical
Treatment of Animals,
PO Box 42516,
Washington,
DC 20015-0516, USA

■ Taub's conviction on one count of the original indictment was overturned on appeal and the remaining 118 counts dismissed — Editor, *Nature*.

The parasites

SIR — Efficient scientific work is increasingly dependent on the rapid availability of laboratory reagents. During the past few years, I have as a routine been placing orders by telefax from remote Finland directly to US dealers and obtained the needed items by express delivery within 2–3 days.

Oncogene Science (Manhasset, New York) responded to my recent order for monoclonal antibodies by advising me to contact its new local distributor in Sweden. When I did so, it turned out that the distributor did not keep the antibodies in stock but had to order them as well.

In a second telefax, I made this clear to Oncogene Science, asking it to avoid further delay of a stopover in Sweden by shipping the antibodies directly to Helsinki. In spite of this, the antibodies were delivered through the distributor in Sweden. The consequence

was a delay of an additional week and a doubling of the price.

It is reasonable that distributors, who keep local stocks of reagents and provide a rapid service, should charge extra to compensate for their costs. But middlemen who add 100 per cent or more to the price only for changing the address labels on the packages and

causing delay are not acceptable. They are just parasites in the scientific community and should be eradicated.

LEIF C. ANDERSSON

Department of Pathology,
University of Helsinki,
SF-00290 Helsinki,
Finland

The collective farm song

КОЛХОЗНАЯ ЗАПЕВКА

Слова А. САДЬНИКОВА

Музыка К. МАССАЛИТИНОВА

Весело, с вадором
Запев (одна)

1. Ве-се-лей иг-рай, гар-мош-ка,
(Двое)

мы сподруж-ко-ю вдво-ём
а-ка-де-ми-ку Лы-сен-ко

Подголосок
Эх.
ве-ли-чаль-ну-ю по-ём.

Хор
Он ми-чу-ринской до-ро-гой
твёр-дой по-ступь-ю и-дёт,

Он ми-чу-ринской до-ро-гой
твёр-дой по-ступь-ю и-дёт,

твёр-дой по-ступь-ю и-дёт,

мор-га-ни . стам, вейс-ма-ни . стам

нас ду-ра-чить не да-ёт.

2. В нашей жизни наступила
Долгожданная весна:
Стала нивам плодородным
Непогода не страшна.
Мы давно уж по науке
Урожай в полях растим.
Мы к земле приложим руки,
Недороды запретим.

3. Академиком Лысенко
Все колхозники горды.
Он во всех краях Отчизны
Учит нас растить сады.
Перестраивать природу
Нам в стране своей пришлось,
Чтоб советскому народу
Благоприятнее жилось.

4. Веселей играй, гармошка,
Мы с подружкой вдвоём
Академику Лысенко
Величальную поём.
Он мичуринской дорогой
Твёрдой поступью идёт,
Морганистам, Вейсманнистам
Нас дурачить не даёт.

1. To the accompaniment of the accordion
My friend and I sing in honour
Of Academician Lysenko*

He follows Michurin's path
With his unerring step
And is not fooled by the followers
of Morgan and Weismann

2. Long-awaited Spring
Has come into our lives
So have fertile fields
That are unafraid of bad weather

Thanks to his teachings
We reap and gather the crop
We have banned bad weather from our fields
By working hard on them

THE document reproduced above, a song on the glories of lysenkoism for peasant parties on collective farms, is further evidence to add to that demonstrating the strength of the malign grip exercised by the biologist T. D. Lysenko on Soviet agriculture earlier this century. At the top is the music and the four verses in Russian with a translation by Barbara Izdebska below. The song was unearthed by Dr Yuri F. Bog-

3. Every member of collective farms
Is proud of Academician Lysenko
He teaches the whole country
How to cultivate gardens

And so there is a 'perestroika' in nature
Across the whole country
which will fructify
of the Soviet nation

4. To the accompaniment of the accordion
My friend and I sing in honour
Of Academician Lysenko

He follows the path of Michurin
With his unerring step
And is not fooled by the followers
Of Morgan and Weismann

danov of the Vavilov Institute in Moscow, and sent on to *Nature* by Professor J. H. Edwards, Genetics Laboratory, Department of Biochemistry, University of Oxford OX1 3QU, UK. Photocopies of the original in full are available from Professor Edwards.

Editor, *Nature*

* Lysenko (1898–1976), Soviet biologist, who had a damaging influence on Soviet biology... (The Macmillan Concise Encyclopedia).

Dr Baltimore says "sorry"

Dr David Baltimore says he had no knowledge of the fabrication of data in a paper in *Cell* of which he was a co-author, says he will work to develop new guidelines for misconduct and apologizes to Dr Margot O'Toole.

A DRAFT report of the investigation conducted by the Office of Scientific Integrity (OSI) of the US National Institutes of Health into the 1986 *Cell* article on transgenic mice has been issued (see *Nature* 350, 262; 1991). I welcome OSI's report because of its completeness and detail. I have now had the opportunity to study OSI's findings and to reflect upon the inquiries and proceedings related to both the paper and the data and experimentation that supported the paper's conclusions.

After an exhaustive review of forensic and scientific evidence, OSI has concluded that certain data contained in the notebooks of one of the paper's authors, Dr Thereza Imanishi-Kari, were falsified and/or fabricated, and that, in relying upon such data, Imanishi-Kari presented false and misleading information to the NIH panel charged with investigating the accuracy of the data and interpretations in the paper. I wish to state that if Imanishi-Kari did falsify data or make misrepresentations, I had no knowledge of the misconduct.

The findings do not undermine either the integrity of the work conducted by my postdoctoral fellow, Dr David Weaver, under my supervision or the reliability of our records. However, the OSI was critical of my response to the mounting challenges raised to the work of Imanishi-Kari, my co-author.

The completion of the NIH investigation has prompted me to make these comments, which will address OSI's observations about my conduct and will share the lessons I have drawn from this experience about the appropriate response to such allegations and the respect and candour that must characterize the partnership between the scientific community and the federal government.

OSI criticizes me for my strong defence of Imanishi-Kari, particularly at the May 1989 hearings before the congressional subcommittee, and for my failure to re-examine Imanishi-Kari's data more critically after serious questions had been raised. I wish to state at the outset that my defence of Imanishi-Kari was not due to any lack of regard for Dr Margot O'Toole, the postdoctoral fellow who first uncovered certain discrepancies in Imanishi-Kari's research. I have tremendous respect for O'Toole, personally and as a scientist, and I have consistently maintained that I believe that her analyses were insightful, her expressions of concern were proper and appropriate, and her motives were pure. Rather, my defence of my co-author was fuelled by my respect for Imanishi-Kari's demonstrated abilities as a scientist, by my

belief that the paper's scientific conclusions were sound, and by my trust in the efficacy of the peer review process.

The study that gave rise to the paper was conducted as a classic collaboration, with each laboratory performing independent research in its particular area. Mutual respect is the bedrock of any professional collaborative effort, and it was a key ingredient in our particular collaboration, because Imanishi-Kari provided the expertise in serology that I lacked — she possessed proven ability in this field.

O'Toole initially made known her concerns to immunologists at Tufts University. Those experts concluded in June 1986 that there was no evidence of deliberate falsification or misrepresentation and characterized the availability of alternative interpretations of the data as "the stuff of science". A later review at Massachusetts Institute of Technology (MIT) reinforced that conclusion. The expert there found that O'Toole had correctly identified a minor error, but explained that the error was too insignificant to warrant a retraction in the light of "a substantial body of other data that is clear and impressive". The MIT report echoed the sentiments of the Tufts reviewers and noted that "other issues raised by O'Toole, which are largely matters of interpretation and judgement, are best dealt with by allowing the scientific process to take its course." I fully expected that this paper, like all others,

would be subjected to the rigours of the scientific peer review process, and that efforts by other laboratories to replicate or extend our findings would ultimately test whether they were correct.

In January 1989, the first NIH panel to investigate the matter concluded, as Tufts and MIT had, that the science in the paper was essentially sound. The report did, however, raise many issues about the way the data for the paper were produced and, in retrospect, it is evident that I gave too much weight to the overall conclusions of the report and did not appreciate that the report might have had the implication that the results had not been obtained as reported.

In 1989, during hearings before the congressional subcommittee investigating the matter, the Secret Service revealed certain preliminary findings regarding its review of Imanishi-Kari's notebooks. At that time, I continued to base my defence of Imanishi-Kari upon the two university reviews, the January 1989 NIH report and my knowledge of her abilities. I now realize that I erred in failing to heed the warnings and that the better course would have been to suspend further comment on the matter until I had a full opportunity to review and digest all of the new information.

In good conscience I feared a rush to judgement, and I accorded my colleague the benefit of every doubt. I now recognize that I was too willing to accept Imanishi-Kari's explanations, and to excuse discrepancies as mere sloppiness. Further, I did too little to seek an independent verification of her data and conclusions. I acknowledge that, for too long, I focused narrowly on the question of whether the paper could stand; what was important to me was that the solid molecular data gathered by my laboratory seemed to lend credence to the serological findings. In other words, as a scientist, my concern was always for the science: is the result correct? Can it be replicated and built upon?

The OSI report raises very serious questions about the veracity of the serological data. I am shocked and saddened by the revelations of possible alteration and fabrication of data. These discoveries are deeply troubling not only because of their impact upon our article, which has been retraced in the light of these revelations, but because such allegations of fraud undermine public confidence in the entire scientific community. Science must be an objective search for truth. It was my

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Baltimore: "temper trust with scepticism".

belief in science and faith in my fellow scientists which led me to set my threshold of suspicion so high.

I wish to state unequivocally that I have never condoned falsity by a scientist. I do not believe it could ever be appropriate to represent that a test that was not performed was in fact completed, or that anything other than the actual results were obtained. Fraud in the laboratory is not only wrong from a moral and legal standpoint, but it impedes the pro-

gress of science, as it makes the review and retesting of hypotheses and conclusions impossible. Deliberate falsification demeans all members of the scientific community because it undermines public trust and confidence in our enterprise.

For their work, scientists are entrusted with public funds. I have come better to appreciate the legitimate role of government as the public sponsor of scientific research and to respect its duty to protect the public

interest and hold the scientific community accountable for its stewardship of public funds. Such accountability can be entirely consistent with the essential objectivity of scientific inquiry.

The case has highlighted the need to conduct our research and review in a manner the public can appreciate, because continued public support is necessary for the continued life of the scientific enterprise and the nurturing of the academic environment in which we enjoy the freedom to experiment and learn. It is only because public support has been translated into federal financial support that scientists have been able to expand dramatically the range of human knowledge and apply this new knowledge to achieve extraordinary practical advances in such fields as medicine and public health. In the light of this creative partnership, I remain firmly committed to the importance of governmental oversight of federally funded projects, and I look forward to continuing to participate in a healthy and necessary dialogue to improve the process.

I have learned from this experience that the accountability to ensure the responsible use of public funds rests not only with each individual scientist but with the scientific and academic communities as whole. Better self-policing and record keeping will facilitate the government's oversight function and may obviate the need for the repeated hearings and investigations that were needed in this case. This matter has also highlighted the need for clear procedures which guarantee the prompt and thorough investigations of allegations, and I hereby commit myself to participate actively in the study and formulation of new guidelines. Questions raised, whether by junior or senior scientists, must be pursued with vigour, and because junior colleagues may be reticent about alleging outright misconduct, it is incumbent upon those more senior to press for a full airing of their suspicions. Any procedures must include the means to protect those who raise concerns from retribution or discrimination. Scientists must ensure that they do not wait too long or set the threshold too high before calling for the application of close scrutiny to ferret out potential falsity. Finally, the questions raised in this investigation have also underscored the need for greater attention to detail in the handling and recording of data, to further effective peer review and to establish an impeccable record for verification of results.

In conclusion, I commend Dr O'Toole for her courage and her determination, and I regret and apologize to her for my failure to act vigorously enough in my investigation of her doubts. I recognize that I may well have been blinded to the full implications of the mounting evidence by an excess of trust, and I have learned from this experience that one must temper trust with a healthy dose of scepticism. This entire episode has reminded me of the importance of humility in the face of scientific data.

David Baltimore

The Baltimore case — a chronology

May 1985: Thereza Imanishi-Kari carries out transgenic mouse experiments at the Massachusetts Institute of Technology (MIT) in an attempt to determine if transplanted foreign genes can affect an animal's own genetic material.

April 1986: Original paper based on the mouse data and finding evidence of genetic changes triggered by the transplanted genes is published in *Cell*, with Imanishi-Kari, David Baltimore and others as authors.

May 1986: Laboratory postdoctoral fellow Margot O'Toole discovers an Imanishi-Kari notebook containing 17 pages that suggested to her that some of the key experiments in the *Cell* paper had never been done; at the request of O'Toole, Tufts University, which is preparing to hire Imanishi-Kari, convenes an *ad hoc* committee headed by biologist Henry Worts to investigate charges.

June 1986: MIT professor Herman Eisen meets O'Toole, Imanishi-Kari and Baltimore to review O'Toole's allegations. His memorandum finds possible minor errors, but no fraud.

October 1986: Contacted by Charles Mapplethorpe, one of O'Toole's former MIT colleagues, National Institutes of Health (NIH) researchers Walter Stewart and Ned Feder begin investigating the case. After they obtain the 17 notebook pages from O'Toole and examine them, they inform NIH officials that they suspect misconduct in the *Cell* paper.

May 1987: NIH Office of Extramural Research begins first inquiry; Tufts committee submits report concluding that there was no deliberate falsification or misrepresentation in the *Cell* paper.

September 1987: After a year of NIH review, Stewart and Feder receive permission to try to publish their 34-page critical analysis of the *Cell* paper, in which they conclude that Imanishi-Kari's experimental records contradict some of the paper's key conclusions. Over the next year *Cell*, *Science* and *Nature* all reject the paper. It is never published.

May 1988: Congressional Investigations subcommittee of Representative John Dingell holds its first hearings, focusing on the response of Tufts and MIT to the O'Toole allegations; Baltimore issues a "Dear Colleague" letter attacking Dingell and asserting that congressional interference "is totally unnecessary".

July 1988: Dingell subpoenas Imanishi-

Kari's laboratory records and turns the notebooks over to Secret Service for analysis.

November 1988: Baltimore and Imanishi-Kari publish a correction in *Cell* indicating that the original paper contained an 'overstatement' of the specificity of BET-1, a key reagent.

January 1989: First NIH investigation ends, finding "significant errors of misstatement and omission... but no evidence of fraud, conscious misrepresentation, or manipulations of data." In a letter, NIH director James Wyngaarden chastises the *Cell* paper authors: "Even though the allegations have been known to you... at least since spring of 1986... you never met to reexamine the data." Such a meeting, he writes, "may have made a full investigation unnecessary."

April 1989: Based on new evidence from the continuing Dingell investigation and subsequent O'Toole findings, NIH reopens the investigation within the newly created Office of Scientific Integrity (OSI).

May 1989: Dingell holds two hearings, at the first of which the Secret Service testify that 20 per cent of a critical notebook is forensically questionable; at the direction of NIH, Baltimore and Imanishi-Kari publish a second correction in *Cell*, giving additional data on the specificity of BET-1.

Summer 1989: Baltimore publishes an article in *Issues in Science and Technology* giving his side of the story and attacking Stewart, Feder and the Dingell staff for unwarranted meddling. "If the sad history of this investigation demonstrates nothing else, it shows that uninformed or malinformed outsiders cannot effectively review the progress of scientific activity," he writes.

May 1990: Dingell holds fourth hearing. Secret Service investigators present additional forensic data showing that Imanishi-Kari's notebook records and purported experiments were "not contemporaneous with respect to time." Findings cast doubt on the data in the second *Cell* correction.

March 1991: Draft report of second NIH investigation reverses previous report, finds "serious scientific misconduct", including data fabrication. Dingell staff announce plans to hold hearings in May on "who-knew-what-when." OSI also states intention to pursue allegations of a cover-up. Baltimore announces that he will retract the *Cell* paper.

C.A.

The making of male mice

Anne McLaren

LAST year saw the discovery^{1,2} of the best candidate yet for the long-sought-after testis-determining gene in mammals (see figure). Its claims rested initially on its loca-

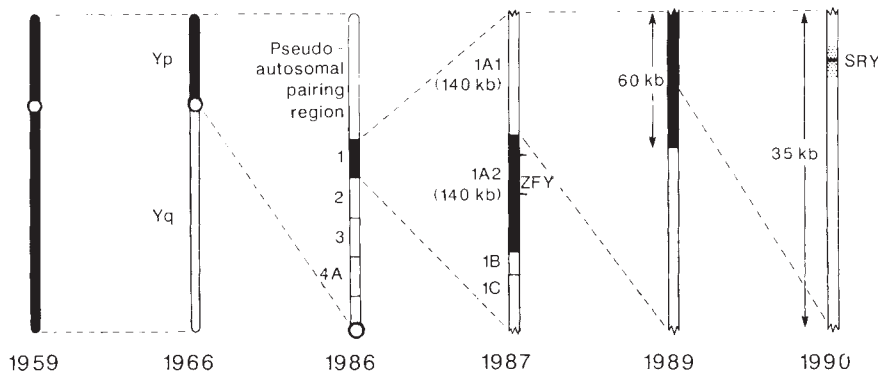
maleness; but certain unsatisfactory features remain. Why only three sex-reversed males? The transgene was inserted in multiple copies: was this a factor? Did the three males

bryonic gonad, but this crucial information is not yet available.

Koopman *et al.* have also produced mice transgenic for the human *SRY* gene. These raise yet more questions. Three integrations were achieved, giving rise to two transgenic lines, and transgene expression in the developing gonads was demonstrated, but no sex-reversal of XX embryos was seen. Would sex reversal have been achieved if more *SRY* transgenics had been made? Or is the human *SRY* product ineffective in the mouse? Or perhaps *Sry* is sufficient to determine maleness, but *SRY* alone is not? Of the four XX human individuals who showed masculinization with only 35 kilobases of *SRY*-containing Y chromosome DNA, all developed testicular tissue but none showed fully normal development of male genitalia⁸.

Most of these questions will probably be answered in the next few months. If mammals turn out to resemble *Drosophila* and *Caenorhabditis* in having a complex cascade of sex-determining genes, the sequence will take longer to unravel. We know that *Sry* must be part of that cascade; what the transgenic results tell us is that the other genes in the cascade, whether they precede or follow *Sry* in the regulatory sequence, must be on the autosomes or on the X chromosome, not on the Y. It seems that *Sry* is indeed *Tdy*, the testis-determining gene on the Y. □

Anne McLaren is in the MRC Mammalian Development Unit, Wolfson House, 4 Stephenson Way, London NW1 2HE, UK.



The hunt for the testis-determining factor from 1959, when the Y chromosome was shown to be male-determining in both mouse and man, to 1990 and the identification of the sex-determining region (*SRY* in humans, *Sry* in mice). For references see the previous News and Views article on the subject⁹.

tion: in both the human and the mouse, it was located within the region that constituted the smallest amount of Y chromosome DNA known to induce masculinization, and in the human it appeared to be the only gene within the relevant 35-kilobase region. It was therefore named *SRY* (human), *Sry* (mouse), for sex-determining region of the Y chromosome.

The candidacy of *Sry* was strengthened by the observation that it was expressed in the developing mouse gonad at the expected time for testis determination; and unlike the previous best bet, *ZFY*, it was not expressed in the developing ovary of mouse embryos in which the testis-determining gene on the Y chromosome (*Tdy*) was known to be defective. Within a few months, further support was forthcoming: *Sry* turned out to be expressed in the gonadal somatic cells previously shown to be responsible for testis determination³, the gene was absent from the *Tdy*-defective mouse mutant⁴, and two sex-reversed XY women were found to have mutations in *SRY* that were not present in their father's gene^{5,6}. It seems that an intact copy of *SRY/Sry* is necessary for testis determination. But is it, in the absence of the rest of the Y chromosome, sufficient?

For mice, the answer is yes, at least sometimes. On page 117 of this issue⁷, Koopman *et al.* describe 11 XX mice transgenic for *Sry* on a 14-kilobase DNA fragment. Three were sex-reversed males, eight were females.

The point is made. *Sry* alone can induce

carry more copies of *Sry* than the eight females? We are not told. Was *Sry* expressed in all 11 transgenics, or did the eight females fail to express it because it was inserted in an inappropriate region of the genome? One of the transgenic females has transmitted the transgene to her progeny, which should allow expression to be examined in the em-

COSMOLOGY

Dark dark matter

Craig J. Hogan

AMONG the suggestions that the Universe is made mainly of invisible material, one of the most predictive is a proposal put forward by Sciama^{1,2}. In this scheme, this 'dark' matter includes neutrinos with a mass of 27.7 ± 0.5 electronvolts (about 0.005 per cent of the electron's mass). The particles are not however entirely dark: they decay occasionally (the lifetime being of the order of 10^{23} seconds) to produce ionizing photons with an energy of 13.8 ± 0.2 electronvolts sufficiently copiously to ionize gas in galaxies today and in the distant intergalactic medium. Until recently these hypothetical decays have eluded direct observational constraints. But the hypothesis has now been dealt a blow by two similar experiments^{3,4}, one reported on page 128 of this issue³, which show that the mass of dark stuff in galaxy clusters does not have the ultraviolet glow which would have been expected if it included such decaying neutrinos.

Massive neutrinos have long been a favourite hypothetical candidate for the dark matter that pervades the Universe. The total number of neutrinos can be predicted from the way they would have interacted with the

hot radiation early in the Universe. So we know that if the one dominant species had a mass of about $100h^2$ electronvolts, where h is the present rate of expansion of the Universe (Hubble's constant, in units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$), it would just suffice to give the Universe the magical critical density, long preferred on aesthetic grounds, which makes space 'flat' (devoid of gravitational curvature) and gives the Universe zero total energy.

A new twist was given to this idea by Sciama, who noticed that if such particles decayed slowly, it would help to solve several other cosmological puzzles by providing a diffuse source of ionizing photons to strip electrons off atoms in intergalactic and interstellar gas. In the interstellar medium, we have an accurate calibration of the electron density along several lines of sight to pulsars, and very simple models of ionization by stars fail to predict this sufficiently uniformly. The distant intergalactic medium is known to be highly ionized, from the absorption spectra of distant quasars, and the source of this ionization has not yet been identified. Sciama showed that both puzzles could be explained

1. Sinclair, A. H. *et al.* *Nature* **346**, 240–244 (1990).
2. Gubbay, J. *et al.* *Nature* **346**, 245–250 (1990).
3. Koopman, P. *et al.* *Nature* **348**, 450–452 (1990).
4. Gubbay, J. *et al.* *Development* **109**, 647–653 (1990).
5. Berta, P. *et al.* *Nature* **348**, 448–450 (1990).
6. Jäger, R. *et al.* *Nature* **348**, 452–454 (1990).
7. Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P. & Lovell-Badge, R. *Nature* **351**, 117–121 (1991).
8. Palmer, M. S. *et al.* *Nature* **342**, 937–939 (1990).
9. McLaren, A. *Nature* **346**, 216–217 (1990).

if cosmic neutrinos decay into ultraviolet photons. He used various arguments to limit severely the range of allowed masses and lifetimes of these decaying particles, thereby making a highly vulnerable, falsifiable theory.

Although highly speculative, interest in the idea mounted over the past year as evidence has appeared from several unrelated directions that at least some neutrinos have mass and decay channels (see Nelson's News and Views article⁵). And the most elegant accepted explanation of the solar neutrino deficit, especially for low-energy neutrinos, involves a conversion between massive electron and muon neutrinos catalysed by the solar plasma. The preferred picture predicts that the more massive of the two has a mass of about 0.001 electronvolts. Laboratory experiments have recently suggested⁵ that a very massive neutrino exists with a small mixing (about 1 per cent) and a mass of about 17,000 electronvolts. If real, this neutrino would need to decay very early in the history of the Universe to avoid dominating its mass and energy budget by a large factor. As there are three neutrino species to play with (under some restrictions, possibly more), and the origin of their masses is poorly understood, the relationship between these results is still obscure, but for just this reason they have served to highlight the central importance of neutrino masses as a way to probe new physics.

Sciama's idea offers another astrophysical window on neutrino properties, as it suggests that neutrinos slowly convert to observable ultraviolet light. Clusters of galaxies represent large concentrations of dark matter — indeed, their dynamics provide some of the strongest evidence that dark matter exists — so careful observations should reveal the photon glow from their steady radioactive decay, if it is as rapid as Sciama requires for it to ionize gas. Until now, it has been difficult to look for this glow because the light has appeared in the ionizing ultraviolet region of the spectrum at which the pervasive cosmic hydrogen is extremely opaque — so opaque that we cannot even see out of a local region of our own Galaxy. The solution to this part of the problem is to look at distant, rapidly receding clusters; the predicted decay line radiation lies at a wavelength only just above the ionizing threshold, so that the small redshift induced by the recession suffices to bring it below the transition to wavelengths at which interstellar gas is transparent. Although the Earth's atmosphere is still highly opaque in this part of the ultraviolet, with spacecraft it is possible to make the observation.

Davidson *et al.* who report in this issue³, used the Hopkins Ultraviolet Telescope on the recent shuttle-borne Astro-1 mission, while Fabian *et al.* used the International Ultraviolet Explorer satellite. Both groups examined selected galaxy clusters (at redshift 0.18 and 0.646 respectively) and found no evidence of spatially extended line radiation

at a level up to 30 times fainter than the simplest models predict.

As always, there are caveats: for example, none of the clusters have precisely determined masses, which leads to uncertainties in the predicted surface brightness even if the neutrino properties are exactly known. Even so, it does not seem possible to make the clusters faint enough to agree with the data. Although small amounts of absorbing neutral gas in the local vicinity of the cluster can absorb the light before it gets redshifted below the ionization threshold, it seems unlikely that the neutral gas would so completely cover the cluster that it would totally obscure the light: to survive in the high-temperature cluster environment, cold gas would need to be highly clumped in clouds.

POPULATION GENETICS

Farming is in the blood

J. S. Jones

WHEN people move, they take their genes with them. On page 143 of this issue¹, Sokal *et al.* show that patterns of inherited variation in modern European populations still reflect the migrations of ancient farmers who spread from the Middle East. Ten thousand years ago, genetic change was driven by economic advance. Remnants of the same process can be seen all over the world today.

Even if the direct line radiation is completely absorbed, the absorbed light eventually gets out as fluorescent line radiation from the atoms, so careful observations should be able to close the remaining loopholes. At present, it seems unlikely that dark matter decay can provide enough photons appreciably to alter the ionization balance in the interstellar medium. □

Craig J. Hogan is in the Departments of Astronomy and Physics, University of Washington, Seattle, Washington 98195, USA.

1. Sciama, D. *Nature* **348**, 617–618 (1990).
2. Sciama, D. *Astrophys. J.* **364**, 549–554 (1990).
3. Davidson, A. F. *et al.* *Nature* **351**, 128–130 (1991).
4. Fabian, A. C., Naylor, T. & Sciama, D. W. *Mon. Not. R. astr. Soc.* **249**, 21r–23r (1991).
5. Nelson, H. N. *Nature* **350**, 461–462 (1991).

expansion into and intermarriage with the native population. The hunter-gatherers of Mesolithic Europe suffered a process of gentrification — or even yuppification — from the east, and it is easy to imagine the complaints of the archers (or stone-throwers) as the incomers with their new-fangled ways, high-technology imports and braying voices moved in on what had been a rural idyll.

Whatever the cultural clashes between old and new Europeans, they were not enough to act as barriers to mating. The survey of Sokal *et al.*¹, of 26 polymorphic systems, including blood groups, enzyme variants and histocompatibility antigens, in over 3,000 places in modern Europe, shows that there is an overall trend in genetic similarity from south-east to north-west (although the ABO blood groups deviate from the general pattern). A map of the spread of agriculture built up from the dates of the latest Mesolithic or earliest Neolithic settlements in each region looks remarkably similar to that of genetic distances.

There is a strong correlation (which is independent of simple geographical position) between the genetic structure of modern populations and the local dates of origin of agriculture.

All this suggests that what Sokal *et al.* call a process of demic diffusion was involved as the wave of advance of farmers spread at about one kilometre a year. As new farms were founded at the edge of the expanding population, the agriculturalists absorbed the genes of the natives. This process began in the Balkans, and was completed thousands of years later on the western fringes of Europe, producing the genetic trends seen

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Early farmers in Europe — spreading the fruit of their labour.

Farming started in Mesopotamia about 9,000 years ago, and covered the whole of Europe within four millennia. Its spread can be followed by the appearance in the archaeological record of artefacts such as decorated beakers. There are two views of the spread of culture — the diffusionist, which emphasizes the passage of new techniques by learning, and the migrationist, which sees mass migrations as the key to ancient economic, social and political change. Genetic patterns suggest that an intermediate process was involved in Neolithic Europe. Economic advance led to population growth of the farmers and hence to their geographical

today. Only one recalcitrant tribe — the Basques — resisted the blandishments of the new technology, and as a group they remain genetically distinct from all other Europeans².

Economic change has often led to human movements whose genetic effects can persist for long periods. The ancestors of Black Americans came to their new home against their will. They brought many genes in addition to those controlling skin colour. Nearly all carried a variant allele at the Duffy blood group locus which is native to west Africa (where it confers protection against malaria) and is absent from whites. But up to a quarter of Duffy alleles in modern American Blacks are of the European type, showing the extent of intermarriage between the two populations over the past 300 years³. Such economic relics persist even in Britain. In 1108, King Henry I moved a group of artisans from the banks of the Tweed to Pembrokeshire, where they set up a weaving industry. Eight hundred years later, their descendants retain a characteristic set of blood group frequencies which differentiates them from their Welsh neighbours⁴.

In King Henry's day, as in modern times, those who could afford it preferred silk to wool. The Silk Road is one of the most ancient of all trade routes and for 2,000 years linked the old Chinese capital of Changan to the Mediterranean. A new survey of haemoglobin variants in a quarter of a million Chinese⁵ shows some clear trends along the Road. There are many abnormal alleles at its western end, suggesting that Mediterranean traders brought with them far more than bills of exchange. Five hundred years ago a shift northwards in the position of the Silk Road from desert into grassland allowed horsemen from the first time to travel fast enough to bring plague into Europe from China. Differential susceptibility to plague by individuals of different blood group may explain why the ABO blood group cline in Europe does not follow the spread of agriculture. This is speculation, but even if it is wrong it emphasizes how much any genetic reconstruction of the past depends on the assumption used: a little bit of natural selection can soon erase the effects of history.

Economic development has led to a dramatic increase in migration over the past century. Anthropologists of the future will face a much more complex pattern of human movement than that which exists at present. Fortunately, it seems certain that today's migrants will be just as punctilious in leaving their genetical visiting cards as were our own ancestors. □

J. S. Jones is in the Department of Genetics and Biometry, University College London, London NW1 2HE, UK.

1. Sokal, R. R., Oden, N. L. & Wilson, C. *Nature* **351**, 143–145 (1991).
2. Sokal, R. R. et al. *Am. Nat.* **135**, 157–175 (1990).
3. Reed, T. E. *Science* **165**, 762–768 (1969).
4. Watkin, I. M. in *Genetic and Population Studies in Wales* (eds Harper, P. S. & Sunderland, E.) 118–146 (Univ. Wales Press, Cardiff, 1986).
5. Li, H. et al. *Hum. Genet.* **86**, 231–235 (1990).

Recycling for a cleaner signal

Frederick J. Raab

OBSERVATION of gravitational waves, the ripples in spacetime emitted by violent cosmic events such as the deaths of stars and the births of black holes, could revolutionize our understanding of astrophysics. Several groups are engaged in attempts to build gravitational-wave observatories based on advanced laser interferometers. Efforts at this frontier have spawned new techniques, such as light recycling and the use of squeezed light to maximize the detectors' sensitivity. A new tool, called dual recycling, has been demonstrated¹ which allows more efficient use of laser light and provides an elegant method of tailoring the bandwidths of these detectors.

A passing gravitational wave causes distances between inertial masses to change by some fractional amount. This effect is measured in an interferometer (see box) by bouncing light beams split from a common laser between mirror-coated test masses in each of two perpendicular interferometer arms, then recombining, or interfering, the beams as they emerge from the arms. If the distances between the masses in both arms are exactly equal, the light recombining at the beam splitter returns towards the laser, leaving a strategically placed photodetector in darkness. The absence of light at this photodetector is referred to as a dark fringe, in contrast to the bright fringe returned to the laser by the beam splitter.

A gravitational wave incident on the detector would upset the balance of the arms' lengths thereby switching some light from the bright fringe onto the photodetector. Because gravitational waves interact weakly with matter, the rich information they carry is preserved as they propagate through space but their influence on detectors is feeble. Predicted signals for the gravitational-wave observatories correspond to fractional dis-

placements of $h = \Delta L / L \approx 10^{-21}$, where ΔL is the difference between the two arm lengths, and L is their average length. Test masses separated by several kilometres will move much less than a nuclear diameter. Laser-interferometer prototypes with a resolution of a few per cent of a nuclear diameter over tens of metres operate today, but further progress is needed.

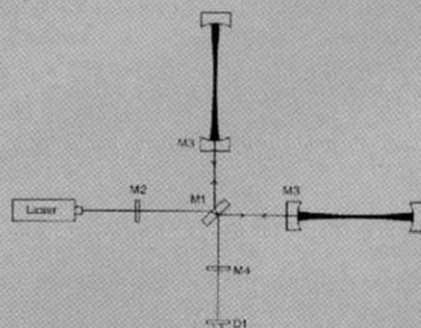
Once the test masses are sufficiently isolated from disturbances attributable to seismic noise and other random forces, the ability to detect a change in the dark fringe fixes the detector's sensitivity. In the 'shot-noise' limit, fundamental fluctuations in photon number inherent in the quantum nature of light make the smallest detectable signal from the interferometer inversely proportional to the square root of the power in the bright fringe.

Light recycling² increases the bright-fringe power obtained with a given laser, in principle achieving the equivalent of a thousand-fold increase in laser power: in broadband recycling (operating over a wide range of gravitational wavelengths) a mirror inserted between the laser and the beam splitter reflects the bright-fringe light back into the interferometer, recycling the photons for another measurement. An alternative technique, resonant recycling, uses a combination of mirrors to switch photons between the two arms in synchrony with the oscillations of a given gravitational wave, allowing the signal to build up over many cycles. In addition to increasing the bright-fringe power, this technique converts the interferometer into a more sensitive, tuned instrument for detecting periodic signals. Dual recycling³ involves recycling both the bright fringe and any signal that would appear at the photodiode, allowing it to build in strength. Although it is conceptually similar to resonant

Laser interferometer with dual recycling

Laser light illuminates the beam splitter (M1) after passing through the power recycling mirror (M2). The beam splitter directs light into two perpendicular interferometer arms where the light is stored between an input mirror (M3 or M3') and an end mirror (unlabelled), bouncing many times between these mirrors (denoted by thick lines). In the delay-line configuration, this light illuminates different spots on these mirrors on each pass; in the Fabry-Perot configuration the same spots are illuminated on each pass, thereby forming an optically resonant cavity with these mirrors. Light returning from the arms interferes at the beam splitter. If both beams have travelled exactly the same distance, all of the light is returned toward the laser; any slight imbalance in the arm lengths causes some light to propagate towards the photodetector (D1). This light is the interfe-

rometer output signal. The power recycling mirror reflects light returned by the beam splitter back into the interferometer.



thereby increasing the circulating power. The signal-recycling mirror (M4) allows the signal to build up in the interferometer in a similar manner before detection at D1.

RÉSUMÉ

Fit connection

Two years after the mapping of the gene which causes a rare form of neonatal epilepsy, a Finnish group reports the localization of the gene for an adult form of the disorder (A.-E. Lehesjoki *et al. Proc. natn. Acad. Sci. U.S.A.* **88**, 3696–3699; 1991). Progressive myoclonus epilepsy of Unverricht–Lindberg type was first described 100 years ago, and is especially common in the Baltic region. Performing linkage analysis with 12 Finnish families, Lehesjoki and colleagues excluded 25 per cent of the genome before tracking down the disease locus to position 22.3 on the long arm of chromosome 21. The most promising candidate gene which maps to the same region encodes the β subunit of the calcium-binding protein S100, expressed mainly in glial cells.

Golden opportunity

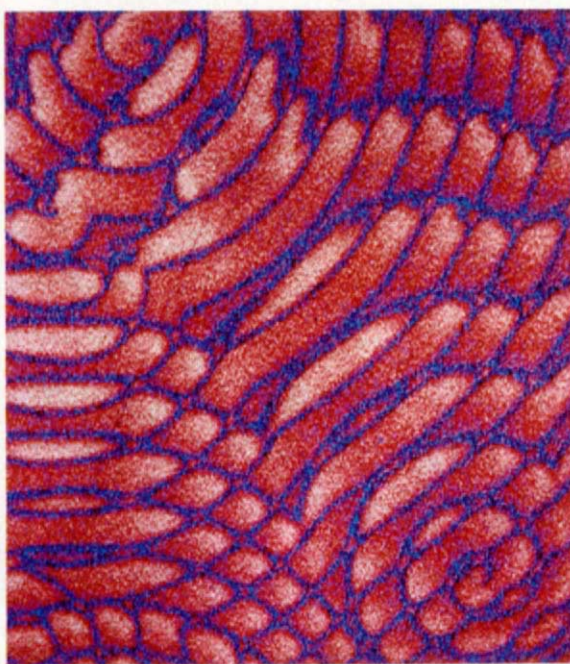
THE discovery in 1984 that certain phospholipids spontaneously arrange themselves into microtubular structures led C. R. Martin and colleagues to see if the use of microporous materials as templates would extend the range of microtubular materials. To the surprise of Martin and C. J. Brumlik (*J. Am. chem. Soc.* **113**, 3174–3175; 1991) these structures are not restricted to organic molecules. Gold can be persuaded to coat the pores of microporous alumina, without blocking them. The alumina can then be etched away to leave tubules with a diameter as little as 0.1 μm and a length of up to 2 μm . One possible application, says Martin, would be to use plugs of gold microtubules, which have a large surface-to-volume ratio, in flowing electrochemical reactors to reduce solutions.

RNA action

MANY of the processes going on inside living cells are carried out by complex machines made up of both proteins and RNA — perhaps the best known case is the translation of messenger RNA by ribosomes, but other more recently fashionable examples include splicing of RNA and protein export. Inspired by these precedents, L. Young *et al.* (*Science* **252**, 542–546; 1991) took another look at the event that creates it all in the first place, transcription of DNA. In eukaryotes, transcription requires, in addition to RNA polymerase itself, a whole array of auxiliary factors, all of which were assumed to be proteins. Not so, it seems. Young and colleagues have found a factor that is essential for transcription by RNA polymerase III in silkworms and that consists, at least in part, of RNA. The number of biological processes that do not require this material shrinks further.

Wave upon chemical wave

As indicated in Ian Stewart's recent News and Views article (*Nature* **350**, 557; 1991), coupled oscillators can offer some spectacular sights. The geometric complexity resulting from two coupled chemical-wave systems, pictured on the right, attests further to the fact. The spiral waves are formed on a polymer (Nafion) membrane loaded with ferroin and immersed in a mixture of the Belousov–Zhabotinsky reagents. The ferroin catalyses this oscillatory reaction, generating wavefronts that propagate on both sides of the membrane. The two sets of waves can communicate by transport of one reagent through the membrane. On page 132, K. Showalter *et al.* show that this interaction can lead to behaviour ranging from irregular patterns to entrainment and phase locking. It is tempting to infer that this kind of phase-sensitive communication could occur across cell membranes. The wavefronts are seen here in blue on both sides of the translucent membrane.



recycling, dual recycling can operate over a wide range of bandwidths.

The new work by K. A. Strain and B. J. Meers¹ demonstrates that dual recycling can improve the broadband low-frequency performance of a particular interferometer configuration which uses optical delay lines to store light in its arms. With this improvement the delay-line interferometer should be as sensitive as a Fabry–Perot interferometer. (Practical constraints on the size of mirrors had previously favoured the more compact Fabry–Perot configuration for detecting gravitational waves at frequencies below a few hundred hertz because the maximum storage time for light in a delay line was too short.) For their demonstration, Strain and Meers built a small-scale broadband recycling interferometer in which the light made a single bounce in each arm. Because the mirrors were not suspended as in a real gravitational-wave detector but were held in rigid mounts, vibrations dominated the displacement noise below 20 kHz. Above this frequency, the displacement noise was shot-noise limited at a level determined by the available bright-fringe power and the very short storage time of light in the arms.

When the signal recycling mirror was added, a sevenfold increase in signal-to-noise ratio was observed. This increase was slightly better than the expected sixfold improvement, a fact which Strain and Meers attribute to the improved quality of the optical wavefront inside the interferometer owing to dual recycling.

Dual recycling also looks promising to

those of us who favour the Fabry–Perot interferometer design. Dual recycling can adapt a broadband recycled interferometer to narrowband operation by adding a single mirror to the output side of the instrument. Adjusting the position of the signal recycling mirror tunes the interferometer to a particular frequency, which might be chosen to correlate with the rotation frequency of a known pulsar. The detector's bandwidth can be varied by adjusting the transmission of the signal recycling mirror. In some cases an intermediate bandwidth may be desirable, trading off some bandwidth for improvements in mode quality within the interferometer.

A very different method for improving interferometer sensitivity in the shot-noise limit is to inject 'squeezed' light into the interferometer at the beam splitter⁴. The presence of squeezed light can alter the stochastic nature of the beam splitting process, reducing quantum fluctuations in the output beam at the cost of increased fluctuations in radiation pressure on the test masses. Although squeezing cannot violate the uncertainty principle, it may allow the quantum limit to be reached with less bright-fringe power. The improvement in precision has been confirmed in a simple interferometer⁵ and the evaluation of squeezing techniques in practical gravitational-wave detectors has begun⁶. For squeezed light to be effective, interferometer losses must be minimized, a situation which also enhances recycling efficiency.

Where will this lead? Gravitational-waves

from sources that can be predicted with confidence, such as coalescing neutron star binaries, should be detectable with long-baseline interferometers using modest broadband recycling factors (about 100) and laser powers (about 60 watts) that should become available in the near future, even without squeezing. The use of squeezed light with broadband recycling configurations should either relax demands for bright fringe power or further extend detector sensitivity. The relative weighting of these techniques in future detectors will hinge on developments in optics, lasers and materials — fields that are now progressing rapidly. As we look to a new frontier in astronomy we should remember a lesson learned from the opening of a

previous frontier, radioastronomy. The Universe was far richer in sources than could previously have been imagined and nobody ever complained that their instruments were too sensitive. □

Frederick J. Raab is at the LIGO Project, California Institute of Technology, Pasadena, California 91125, USA.

1. Strain, K. A. & Meers, B. J. *Phys. Rev. Lett.* **66**, 1391–1394 (1991).
2. Drever, R. W. P. in *Gravitational Radiation*, Les Houches 1982 (eds Deruelle, N. & Piran, T.) 321–338 (North Holland, Amsterdam, 1983).
3. Meers, B. J. *Phys. Rev. D* **38**, 2317–2326 (1988).
4. Caves, C. M. *Phys. Rev. D* **23**, 1693–1708 (1981).
5. Xiao, M., Wu, L. & Kimble, H. J. *Phys. Rev. Lett.* **59**, 278–281 (1987).
6. Gea-Banacloche, J. & Leuchs, G. J. *opt. Soc. Am.* **B4**, 1667–1676 (1987).

Architecture with a difference

David J. Filman and James M. Hogle

WITH the description by Rossmann and colleagues of the structure of canine parvovirus at 3.25 Å resolution¹, we have the first example of the architecture of a virus with a single-stranded DNA genome. Although the protein coat (capsid) of canine parvovirus includes a core structural motif that has frequently been observed in RNA viruses, the arrangement of the motif differs considerably from anything known previously. The new structure provides several insights into the assembly and biological properties of simple viruses.

The architecture of small viruses is dictated by the need for the 'blueprint' for a large and elaborate protein coat to be encoded by a relatively small genome. This requirement is satisfied by constructing the coat from several copies of small protein subunits which pack together in a regular arrangement to form a particle either with helical symmetry (for filamentous viruses) or icosahedral symmetry (for 'spherical' viruses). An icosahedrally symmetrical capsid is constructed from 60 copies of some repeating unit, with one or more proteins in each unit. In most icosahedral viruses the capsid proteins contain a wedge-shaped core motif (consisting of an eight-stranded antiparallel β barrel), with each motif representing protein of relative molecular mass about 20,000 (M_r 20K) (Fig. 1).

Indeed, among the published structures of small icosahedral viruses the only exception to this generalization is MS2, a small RNA bacteriophage, whose coat proteins form dimers that are remarkably similar to the α1 and α2 domains of the class I major histocompatibility antigen². But a shell constructed of 60 copies of a minimal core motif is large enough only to encapsidate a genome encoding little more than the coat protein itself. This is the case with satellite tobacco necrosis virus which depends on a helper virus to supply other proteins necessary for replication³. Viruses which code for their own re-

pliative enzymes build larger shells either by forming the repeating unit from multiple copies of a single subunit (for example 180 copies of one kind of subunit in the 'T=3' plant viruses^{4,5}), by forming the repeating unit from several chemically distinct wedge-shaped protein domains (for example the picornaviruses^{6,7} and comoviruses⁸), or by using a single, much larger, protein subunit.

The canine parvovirus (CPV) particle has an outer diameter of 250 Å, and so is intermediate in size between satellite tobacco necrosis virus (180 Å) and the T=3 plant viruses and picornaviruses (300 Å). Its 5,000-base DNA genome is roughly the same size as the RNA genomes of the T=3 plant viruses, and encodes the capsid proteins and two nonstructural proteins which

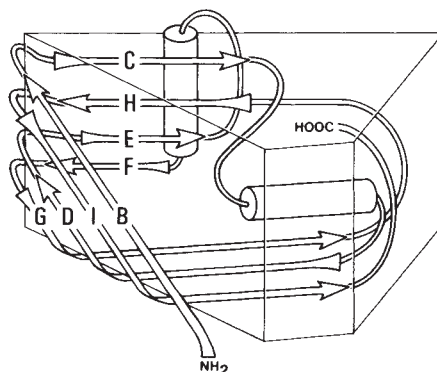


FIG. 1 Schematic representation of the wedge-shaped eight-stranded antiparallel β barrel core motif shared by the major capsid proteins of several icosahedrally symmetrical viruses. The proteins which contain this common folding pattern have structurally similar cores, but exhibit great diversity in the loops which connect the β strands, and in their N and C extensions. Individual β strands are shown as arrows, and are labelled alphabetically. The flanking helices, which are indicated by cylinders, are not absolutely conserved, but are common to many virus structures, including that of canine parvovirus.

function as transcription regulators. Early studies showed that the virion, the infectious particle, is composed of variable numbers of three large proteins, VP1 (M_r about 85K), and VP2 and VP3 (each of M_r about 65K). These observations were difficult to reconcile with the need for an icosahedral capsid to contain a multiple of 60 subunits, until sequence analyses revealed that VP1 is derived by an alternative splice of the capsid message (adding an additional 20K domain to the N terminus of VP2), and that VP3 is derived by post-translational cleavage of 15–20 residues from the N terminus of VP2. Thus, the virion contains exactly 60 copies of the common minimal 'shell-forming' sequence of VP3. Remarkably, however, this minimal sequence is more than twice as large as the capsid-forming subunits observed in the other virus structures, though it contains only one wedge-shaped core motif. The unusually large size of the protein (and hence the ability to form a 250 Å shell from only 60 copies of a single protein) results from the presence of several large loops connecting the strands of the β-barrel core, including an especially large loop (231 amino acids) connecting the G and H strands (Fig. 1).

The arrangement of capsid protein subunits in the shell of CPV is not unexpected, but it represents a previously unseen mode of icosahedral packing. Although an infinite variety of choices is available for selecting the shape of each of the 60 repeating units which tessellate an icosahedral surface, among the simplest choices are the 'triangular' wedge bounded by a fivefold and two threefold axes of symmetry, and the quadrilateral 'kite-shaped' wedge bounded by a fivefold, threefold and two twofold axes of symmetry (Fig. 2, left). In the known icosahedral virus structures, the subunits are fairly compact and, to a good approximation, the 'geographical' limits of the protein subunits happen to coincide fairly well with the boundaries of either the triangular or the kite-shaped choices of icosahedral unique volume. In the T=3 icosahedral plant viruses, each triangular area contains three chemically identical copies of the capsid protein (labelled A, B and C in Fig. 2); in the picornaviruses, the biologically relevant protomer includes three structurally similar but chemically distinct copies of the core motif (labelled VP1, VP2 and VP3), arranged to occupy approximately the kite-shaped area. Satellite tobacco necrosis virus is the one-domain analogue of the T=3 viruses, with each capsid protein occupying the triangular asymmetric unit (Fig. 2, top right). Surprisingly, in CPV the capsid protein occupies the kite-shaped asymmetric unit (Fig. 2, bottom right), and thus it can be thought of as the first example of a single-domain analogue of the picornavirus architecture.

The CPV structure includes several other unusual features. One is the presence of strong electron density corresponding to at least 11 nucleotides of icosahedrally ordered DNA (per protein subunit) bound to the

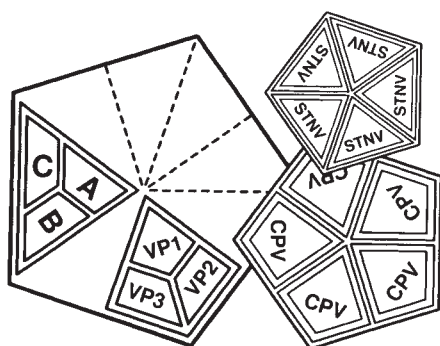


FIG. 2 The architectures of small icosahedrally symmetrical viruses illustrate some of the ways to form a closed protein shell from 60 identical copies of a simple shape. The 'triangular' choice of repeating unit is occupied by one capsid protein subunit in satellite tobacco necrosis virus (STNV), and by three chemically identical subunits (A, B and C) in the T=3 icosahedral plant viruses. The 'kite-shaped' repeating unit is occupied by a single large subunit in canine parvovirus (CPV), and by a protomer that includes three chemically distinct large subunits (VP1, VP2 and VP3) in the picornaviruses. Five copies of either simple shape can form a pentagon (as shown); and 12 pentagons can be arranged to form a closed surface.

inner surface of the capsid. Similarly well-ordered nucleic acid structure has been observed only once before, in bean pod mottle virus⁸. Being able to see a protein–nucleic acid complex is valuable to our understanding of the recognition process, both because there are few crystalline examples of biologically relevant complexes, and because the recognition of virus-specific nucleic acid is an important mechanism in the control of viral self-assembly.

A second striking feature is the presence of a hollow tube at each fivefold axis, extending from the inside of the particle to the outside. The tube, which is at least 8 Å in diameter throughout its length, is formed by the association of five copies of a two-stranded β sheet contributed by residues in the loop connecting the D and E strands at the narrow end of the β-barrel core. The presence of strong electron density on the inside of this tube in virions, but not in naturally occurring 'empty' particles, the observation that the N-terminal domain of VP1 is susceptible to proteolysis in virions but not in empty capsids, and the presence of a well-conserved sequence of small amino acids in the polypeptide strand which connects the N-terminal domain of VP1 to the β-barrel core, all suggest that the cylindrical structure furnishes a route for the externalization of up to 12 copies of this domain when a full

complement of viral DNA is present.

Finally, the new structure provides a framework for investigating the determinants of host range and antigenicity in canine parvovirus. Because only four changes in the capsid protein sequence (and three silent mutations) are sufficient to permit the related feline panleukopenia virus to grow in dogs, CPV is an appropriate model system for investigating the mechanisms of host-range restriction. In addition, because there is no evidence for its existence as a pathogen earlier than 15 years ago (it is

now endemic among wild and domestic dogs), CPV is an excellent system for addressing the related question of how viruses nominally specific for one host become adapted to infect another. Given the recent identification of a variety of novel pathogens in humans, this question is of more than casual interest. □

David J. Filman and James M. Hogle are in the Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, California 92037, USA.

BLACK HOLES

Bigger and better

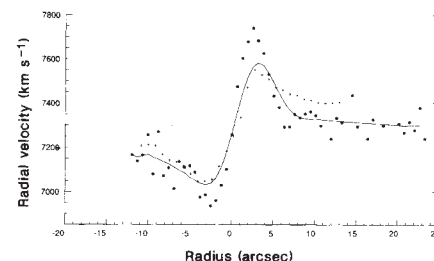
James Binney

A BLACK hole more massive than any so far identified seems to lie at the heart of the distant galaxy NGC6240 — or so suggest Bland-Hawthorne *et al.* in the 10 April issue of the *Astrophysical Journal*, on the basis of high-resolution studies of the dynamics of gas in the galaxy.

Astronomers have long believed that black holes with masses as large as 10^9 solar masses ($M_\odot$) lurk at the centres of many large galaxies, because in the conventional model of a quasar — an object no bigger than the Solar System yet often bright enough to be seen right across the Universe — the system is powered by the accretion of gas onto a massive black hole. Actually finding one of these black holes has proved extremely difficult, however. A black hole can be detected only through its gravitational field, which in effect means by studying the motion of gas and stars near it. At a separation of about 50 parsecs (150 light-years), the speed of a star on a circular orbit around a $10^9 M_\odot$ black hole becomes comparable to the typical speed, about 300 km s^{-1} , of a star at the centre of a giant galaxy. So to detect a $10^9 M_\odot$ black hole by its effect on the motion of stars in the nucleus of a galaxy, one has to be able to resolve structure within the galaxy's central 100 parsec. For the nearest appropriate galaxies, numbers 31 and 32 in Messier's catalogue, this distance translates to 36 seconds of arc, so measurements of the required resolution are readily obtained from the ground in the optical band. But by Murphy's law these nearest galaxies do not contain super-massive black holes, and the evidence that they harbour less massive black holes is still not firm (though it is fairly convincing^{2,3} in the case of Messier 32).

Twelve years ago a group at Caltech announced⁴ the discovery of a black hole with mass of about $5 \times 10^9 M_\odot$ at the centre of one of the nearest giant radio galaxies, Messier 87. This galaxy is roughly thirty times further away than Messier 32, so the discovery involved resolving structure only a few arc-seconds across. Since that pioneering work a debate has raged as to whether the inevitable uncertainties in such small-scale detail allow

the measurements to be interpreted without invoking a central massive object. The jury is still out on this one, although the case for a black hole is now a strong one. Bland-Hawthorne *et al.*¹ have been observing a galaxy six times further away still than Messier 87, and argue that it harbours an even more massive black hole: $M > 10^{10} M_\odot$. Their



The rotation curve for Disk 2 in the double-nucleus galaxy NGC6240. The steepness of the curve could indicate the presence of an ultramassive black hole in the vicinity.

work differs from that of the Caltech group and their successors in focusing attention on the dynamics of gas rather than stars — Bland-Hawthorne *et al.* used a Fabry–Perot etalon spectrometer to determine line-of-sight velocities of the galaxy's hydrogen from doppler shifts in the Hα emission line.

In a quiescent system, gas soon settles to an orderly disk of material on effectively circular orbits and provides the ideal probe of the local gravitational field. (Indeed, it is precisely by studying gas disks around spiral galaxies that astronomers have concluded that galaxies are enveloped in much more massive 'dark halos'.) However, NGC6240, the galaxy studied by Bland-Hawthorne *et al.*, is by no means quiescent. In fact it is generally agreed to be *two* galaxies in collision. In particular, it has a double-humped nucleus and Bland-Hawthorne *et al.* interpret the kinematics of its gaseous component in terms of two disks rotating in different planes.

Disk 1 appears to rotate like many another galactic disk: as one looks further from the nucleus, the rotation speed rises approxi-

1. Tsao, J. *et al.* *Science* **251**, 1456–1464 (1991).
2. Valegård, K., Liljas, L., Fridborg, K. & Unge, T. *Nature* **345**, 36–41 (1990).
3. Liljas, L. *et al.* *J. molec. Biol.* **159**, 93–108 (1982).
4. Harrison, S.C., Olson, A.J., Schutt, C.E., Winkler, F.K. & Bricogne, G. *Nature* **317**, 368–373 (1978).
5. Abad-Zapatero, A. *et al.* *Nature* **286**, 33–39 (1980).
6. Hogle, J. M., Chow, M. & Filman, D. J. *Science* **229**, 1358–1365 (1985).
7. Rossmann, M. G. *et al.* *Nature* **317**, 145–153 (1985).
8. Chen, Z. *et al.* *Science* **245**, 154–159 (1989).

mately linearly with radius before flattening off at a value that remains constant as far out as it can be followed. The 'rotation curve' of Disk 2, shown in the figure, is very different. Near its centre the line-of-sight velocity changes by 800 km s^{-1} in 5 arcseconds (about 2.4 kiloparsec). In fact this change may be even steeper than the figure suggests, as Bland-Hawthorne *et al.* could not have spatially resolved a more abrupt change. In any event, if one assumes that material at the peaks of the rotation curve is on circular orbits, one can estimate the mass within 2.5 arcseconds required to hold it on such orbits; nothing less than $4.5 \times 10^{10} M_{\odot}$ will do.

This is, by any standards, a lot of mass in a small space, but what makes it more remarkable is how little light is coming from the same region. This region does not include the galaxy's double-humped nucleus, and even after making generous allowance for absorption of its luminosity by dust, with this mass estimate it contains more than six times as much mass per unit luminosity as a normal stellar population. Thus the straightforward interpretation of the rotation curve in the figure suggests that near the centre of Disk 2 NGC6240 is twice as dark as the famous core of Messier 87. If this darkness is caused by adding a black hole to a normal stellar population, the hole must weigh more than $3 \times 10^{10} M_{\odot}$, much more than any conceivable black hole in Messier 87.

The key question is: can one safely interpret the peaks in the rotation curve as the local speed of a circular orbit? One striking feature of the figure is the extremely rapid decline in the rotation velocity just outside the peak. This decline is faster than the form, $v \propto 1/r^{1/2}$, characteristic of rotation about a point mass. Such a steep fall-off in velocity is most naturally explained in terms of a stellar bar across the middle of the galaxy; the shapes of orbits in a barred gravitational field can change rapidly from elongated to round, and this gives rise to steep gradients in line-of-sight velocities when the system is viewed from certain angles⁵. Furthermore, numerical simulations of mergers⁶ show that the old stellar components of merging galaxies tend to develop strong bars whose gravitational fields deprive gas of its angular momentum. The gas then plunges to the system's centre on highly elongated orbits. Could the observations of Bland-Hawthorne *et al.* be neatly explained by such a model without invoking a massive black hole? In any event, the search for these objects must go on. □

James Binney is in the Subdepartment of Theoretical Physics, University of Oxford, 1 Keble Road, Oxford OX1 3NP, UK.

Playing a molecular accordion

Michael Cates

BIOLOGICAL membranes, such as cell walls, are composed mainly of lipid bilayer. In red blood cells, the stability and shape of the membrane is partly controlled by coupling to an adjacent network of polymer molecules (the cytoskeleton), attached to the membrane by anchor groups. The physical

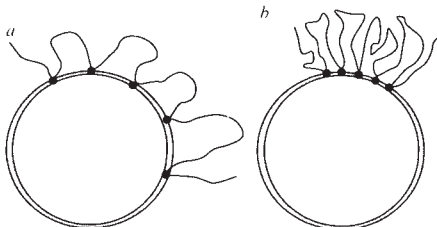


FIG. 1 Adsorbed state of polymer and lipid bilayer. a, Good solvent; b, poor solvent.

properties of purified model membranes, which can be made to form small spherical shells called liposomes, have been studied extensively¹. Now, Ringsdorf *et al.*² have studied the adsorption of a synthetic polymer (with suitable anchor groups) onto liposomes, and have shown that a reversible folding of the attached polymer can occur under changes in temperature; they call their system a "molecular accordion" (Fig. 1). The authors draw a parallel between this effect and the reversible changes of shape that occur in the cytoskeleton of red blood cells during cell motion.

The system the authors studied consists of a high-molecular-weight, water-soluble polymer, poly-*N*-isopropylacrylamide (PNIPAM), to which aromatic side groups (the anchor groups) are attached every 200 monomers or so along the chain. The authors added this to a solution of unilamellar liposomes. The state of adsorption and folding of the chain was monitored by a fluorescence technique, which provides data on what fraction of the anchor groups are close to another such group. The fraction is high when the polymer is dissolved in water (without added liposome) since in this case the polymer forms a micellar structure (Fig. 2) in which the anchor moieties are clustered together to avoid unfavourable contact with water. On addition of liposomes (made of dimyristoyllecithin, DMPC), the fluorescence signal decreases dramatically, indicating that anchor groups are well separated — as with the adsorbed-layer structure of Fig. 1a.

The molecular-accordion effect is then brought about by increasing the temperature of the system. At around 32°C , PNIPAM has a lower critical solution temperature: water is a good solvent for the polymer below this temperature, and an isolated coil of pure PNIPAM (without any anchor groups) will form an expanded structure to maximize its contact with the solvent. At higher temperatures, however, such contacts are unfavourable and the coil collapses. This coil-globule

transition is ubiquitous in simple polymers (although in many systems the dependence on temperature is reversed). A similar transition is often seen in proteins, although the chemical heterogeneity of the chain means that the effect is a more complex one. Ringsdorf *et al.* exploit the phenomenon by raising the temperature of the combined polymer-liposome system (Fig. 1a). The collapse of the chain causes the anchor groups to move inwards, increasing the number of anchor-anchor contacts and enhancing the fluorescence signal (Fig. 1b). The whole process is reversible, so that the adsorbed polymer can be expanded and contracted repeatedly by cycling the temperature — like playing an accordion.

This is certainly a neat experiment, which illustrates an elegant if straightforward piece of physics. But what is its significance for biology? The new results of Ringsdorf *et al.* follow earlier work in which it was shown that the equilibrium shape of a liposome can be strongly altered by adding adsorbed polymer^{3,4}. Despite this, there is no evidence in the PNIPAM/DMPC system that the liposomes actually 'dance to the accordion' by undergoing sympathetic changes in shape (the anchor groups could simply slide together within the bilayer, which acts as a two-dimensional liquid). The authors sug-

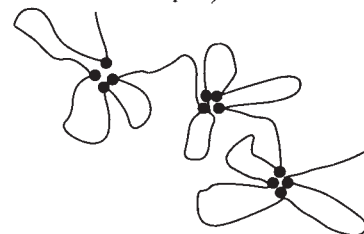


FIG. 2 Micellar state of a free chain containing anchor groups.

gest that this might be achieved by choosing larger, more flexible liposomes and by cross-linking the adsorbed polymer into a network. In any case, the numerous shape changes of red blood cells suggest an exquisite dependence of the cytoskeletal protein network on physiological conditions; it would be surprising if such subtlety were related to so gross a conformational change as a coil-globule transition. We do not yet know whether the molecular accordion and the red blood cell cytoskeleton are really playing the same tune. □

Michael Cates is at the Cavendish Laboratory, University of Cambridge, Madingley Road, Cambridge CB3 0HE, UK.

1. Bland-Hawthorne, J., Wilson, A. S. & Tully, R. B. *Astrophys. J.* **371**, L19–L22 (1991).
2. Tonry, J. L. *Astrophys. J.* **322**, 632–642 (1987).
3. Dressler, A. & Richstone, D. O. *Astrophys. J.* **324**, 701–713 (1988).
4. Sargent, W. L. W. *et al.* *Astrophys. J.* **221**, 731–744 (1978).
5. Gerhard, O. E. & Vietri, M. *Mon. Not. R. astr. Soc.* **223**, 377–389 (1986).
6. Barnes, J. & Hernquist, L. *Astrophys. J.* **370**, L65–L68 (1991).

1. Leibler, S. in *Statistical Mechanics of Membranes and Surfaces* (eds Nelson, D., Piran, T. & Weinberg, S.) 45–103 (World Scientific, Singapore, 1989).
2. Ringsdorf, H., Venzmer, J. & Winnik, F. *Angew. Chem. Int. Ed. Engl.* **30**, 315–318 (1991).
3. Decher, G. *et al.* *Angew. makromol. chem.* **166/167**, 71–80 (1989).
4. de Gennes, P. G. *J. phys. Chem.* **94**, 8407–8413 (1990).

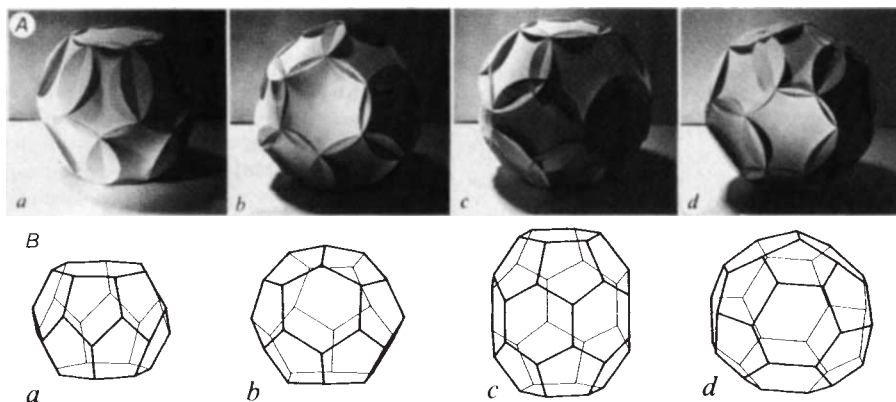
Circularly covering clathrin

Ian Stewart

MATHEMATICIANS often take inspiration from nature, the latest case in point being the problem of covering a sphere by identical circular disks. In *Journal of Molecular Biology*¹, with details to appear elsewhere²,

tetrahedron, and twelve at the vertices of a regular dodecahedron.

The best possible solutions are known only when the number of disks is at most 14 (ref. 3). For greater numbers, conjectured optimal



A, Cardboard models of the covering of a sphere by disks of equal size. *a* and *b*, covering by 16 disks; *c* and *d*, covering by 20 disks. *a* and *c* are the conjectured optimal solutions for 16 disks³ and 20 disks⁴; *b* and *d* are the better solutions offered by Tarnai's study of clathrin cages. B, Stick polyhedra of equal edges, each of which corresponds topologically to the similarly labelled photograph in A. *a*, 'Heptagonal drum' of 2 individual heptagons and 2 rings of 7 pentagons. *b*, Cage polyhedron of 12 pentagons and 4 hexagons, tetrahedrally arranged. *c*, The 'hexagonal barrel', a cage polyhedron of 2 rings of 6 pentagons, 1 ring of 6 hexagons and 2 individual hexagons. *d*, The 'tennis ball', a cage polyhedron of 2 curved strips of 4 hexagons, and 1 space loop of 12 pentagons analogous to the seam of a tennis ball. (Reproduced from ref. 1.)

Tibor Tarnai announces that several arrangements widely conjectured to be the best possible can in fact be improved. The improvements are derived from the observed form of biological structures known as 'coated vesicles'.

First, the covering problem. An alien race, the Yuppi, are installing a network of cellular telephones on a distant world. The planet has no open bodies of water, and may be considered a perfect sphere. A certain number of radio stations must be placed on the planet so that every point is within receiving distance of at least one station. Because the Yuppi place great store on cost-effectiveness, and stronger radio receivers are more expensive, they wish to minimize that distance. In more orthodox language, if a sphere is to be covered by a number of identical circular disks, how should they be placed to make their common radius as small as possible?

For small numbers of disks the problem is easily solved. For example one disk can be placed anywhere, and its radius should be half the sphere's circumference, so that it wraps itself round the entire sphere. Two disks should be diametrically opposed, like north and south hemispheres. Symmetric arrangements tend to occur; thus four disks should be placed at the vertices of a regular

coverings have been published only for 16 disks³ and 20 disks⁴ (see figure, Aa and Ac).

Enter the coated vesicles. These have an external lattice structure known as clathrin. R. A. Crowther and colleagues⁵ noticed that several common clathrin cages possess 12 PLANETARY ATMOSPHERES

Jupiter's stratosphere mapped

Peter J. Gierasch

OUR knowledge of Jupiter (and the other outer planets) is largely confined to a two-dimensional view at the height of the visible clouds. Motions, patterns, coloration and chemical composition are richly variable at this level, but an understanding of the system will require information from the third dimension. In *Science*, Orton and 14 colleagues¹ present maps of thermal emission from stratospheric levels which will help alleviate the difficulty. The planet was monitored for a decade, a full jovian annual cycle. In spite of the planet's small (3°) obliquity, seasonal effects appear. In addition, there is exciting evidence for variability on a short timescale (only months) which probably indicates upward propagation of disturbances from within or below the visible clouds.

The maps were made at a wavelength of 7.8 μm at the NASA Infrared Telescope Facility on Mauna Kea. At this wavelength, emission is from the middle stratosphere in thermodynamic equilibrium, about 60 km

above the visible clouds (Fig. 1). The emitting gas is methane, which is uniformly mixed in the atmosphere of Jupiter. Variations in emission are due to temperature variations in the atmosphere (composition 90% hydrogen, 10% helium and 0.25% methane by number). Horizontal temperature gradients can be produced dynamically by disturbances that penetrate upward from below or by local *in situ* variations in solar heating due to absorption by variable hazes or trace gases. In the latter case the thermal response is governed by a radiative time constant of several years. In the former case fluctuations can be much more rapid, limited only by the wave propagation characteristics of the atmosphere and the rapidity of the excitation processes, which could be as vigorous as terrestrial thunderstorms if they originate in the jovian water clouds, which are very likely to have latent heat effects of the same order as on Earth.

The same thing happens in the sphere-covering problem. The arrangement of the disks can be represented by a polyhedron whose vertices are placed at, or radially in line with, their centres; and when there are more than 14 disks, the best known coverings all correspond to polyhedra with 12 pentagonal faces, the remainder being hexagonal. In view of this, Crowther *et al.* suggested that clathrin cages might provide optimal solutions to the sphere-covering problem. For example, the conjectured solution for 20 disks is identical to the clathrin cage known as the 'hexagonal barrel' (see figure, Bc).

Tarnai investigates this suggestion in three cases: 16, 20 and 32 disks. For 16 and 20 disks it turns out that the clathrin cages do not correspond to the arrangements conjectured to be optimal by mathematicians. Instead, they provide *better* solutions to the sphere-covering problem (see figure, A). Thus for 20 disks the improved solution corresponds not to the hexagonal barrel, but to a clathrin cage known as the 'tennis ball'. The angular radius of the circular disks improves from the conjectured 30.5° to 29.6°. For 16 disks the improvement is from 33.5° to 32.9°. But when the number of disks is 32 the clathrin structure does agree with the conjectured optimal solution — the truncated icosahedron or 'soccer ball'.

Although mathematicians often take inspiration from nature, usually they expect it to provide problems, not answers. Not for the first time, nature has beaten them at their own game. □

Ian Stewart is in the Mathematics Institute, University of Warwick, Coventry CV4 7AL, UK.

1. Tarnai, T. *J. molec. Biol.* **28**, 485–488 (1991).
2. Tarnai, T. & Gáspár, Z. *Mathl. Proc. Camb. phil. Soc.* (in the press).
3. Tóth, F. *Studia Sci. Math. Hungar.* **4**, 225–247 (1969).
4. Jucović, E. *Mat.-Fyz. Casopis. Slovensk. Akad. Vied.* **10**, 99–104 (1960).
5. Crowther, R. A., Finch, J. T. & Pearse, B. M. F. *J. molec. Biol.* **103**, 785–798 (1976).

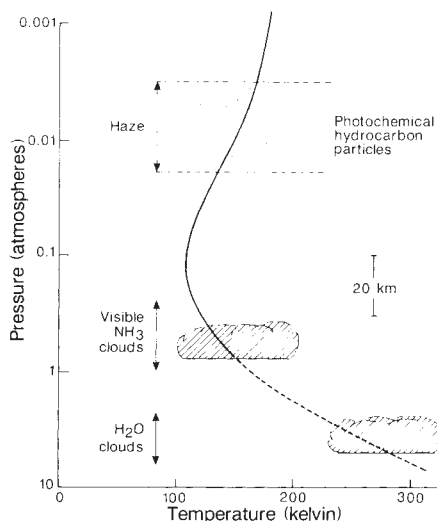
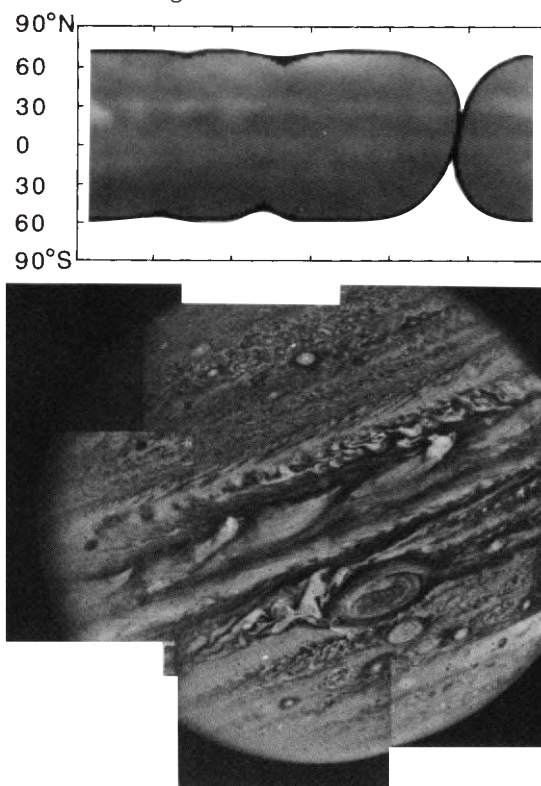


FIG. 1 Vertical structure of Jupiter's atmosphere. The solid line indicates a mean temperature profile as determined by infrared remote sensing. The dashed line is an approximately adiabatic extrapolation through the water clouds, where much dynamical activity is believed to be seated. This level is determined by the water-to-hydrogen ratio (still not well known and probably regionally variable) and the temperature–pressure relation along the adiabat. The maps by Orton *et al.* refer to stratospheric level within the photochemical base. The haze particles are small and do not radiate to any important extent at $7.8\ \mu\text{m}$, but they absorb sunlight and thereby affect the gas temperature.

FIG. 2 Jupiter in visible light (bottom) and mapped at $7.8\ \mu\text{m}$ (top). A mosaic of Voyager images shows the variety of dynamical activity at the ammonia cloud level on Jupiter. The map of infrared brightness by Orton *et al.* is indicative of the gas temperature about 60 km higher.



nomena of both types. On the slow, seasonal timescale there is a north–south temperature asymmetry that flips sign with the season in the expected manner. In addition, there is an unexpected oscillation at low latitudes whose period is half the orbital period. Two temperature maxima at about $\pm 20^\circ$ latitude appear and disappear, symmetrically and in step, during this cycle. The amplitude is approximately 2 K, which is of the same order as the global seasonal contrasts. Since the frequency of this phenomenon is different from that of the direct solar forcing, it must be due to a nonlinear dynamical process, most probably seated in the deep atmosphere.

On shorter timescales the new data are, unfortunately, incomplete — but also tantalizing. The maps show longitudinal structure at a variety of length scales, with a particularly strong signal at a wavenumber of about 11 at a latitude of about 20°N (Fig. 2). The structure varies on timescales as short as a month, and future observations at shorter time intervals will be needed to characterize the changes fully. This surprisingly short timescale (by jovian standards) must mean that these disturbances are attributable to dynamical effects that penetrate all the way from the visible cloud level (or deeper) into the stratosphere. Within the stratosphere itself, there is no obvious way for producing such variability locally: radiative heating is weak, and horizontal temperature gradients are too small to cause instabilities.

On both timescales, the new data point to the equatorial zone, between ± 20 degrees of latitude, as being of special interest to an understanding of the jovian general circulation. This zone includes the $100\ \text{m s}^{-1}$ equatorial jet stream and the strong shears at its edges. Previous observations, both in the visible and in the infrared², have also indi-

cated particularly vigorous activity in this region. This zone and the Great Red Spot³ seem to be the two regions on Jupiter where a much needed three-dimensional picture of the circulation might emerge in the near future. Both of the regions are characterized by geometrically large dynamical systems with strong flows and a vertical dependence that may be amenable to observation and analysis. □

Peter J. Gierasch is at the Center for Radio-physics and Space Research, Space Sciences Building, Cornell University, Ithaca, New York 14853, USA.

1. Orton, G. S. *et al.* *Science* **252**, 537–542 (1991).
2. Gierasch, P. J., Conrath, B. J. & Magalhães, J. A. *Icarus* **67**, 456–483 (1986).
3. Dowling, T. E. & Ingersoll, A. P. *J. Atmos. Sci.* **46**, 3250–3278 (1989).

Green music

EVER since Darwin tried playing a bassoon to a sensitive plant, the effect of speech and music upon the vegetable kingdom has been a persistent if disreputable area of research. There have even been claims that plants react to musical style — preferring classical to jazz, for example, or absorbing more water when exposed to rock music. Daedalus now has an explanation for such findings.

A green plant, he points out, is a pump. It extracts water from the soil, pumps it upwards as sap, and evaporates it from the leaves. Like all biological fluids, plant sap has a non-newtonian rheology. Accordingly, if placed in an asymmetric opening (such as the stomatal pore of a leaf), and exposed to an asymmetric waveform, its oscillatory flow will not average to zero. It will be pumped either into or out of the leaf. Evolution has presumably arranged matters so that it is pumped outwards by naturally occurring waveforms, such as those generated by the flexing of the leaf in the wind. But a thin, flexible leaf is an ideal microphone. It should also be sensitive to asymmetric sonic waveforms, like the percussive components of rock music.

So DREADCO biologists are exposing various crop plants to asymmetric waveforms, and observing their growth and transpiration rates as a function of waveform and frequency. A plant with large leaves, like rhubarb, will be most affected by low frequencies, while those with small leaves, like beans, will respond better to the upper registers. Elongated leaves, for example those of conifers, wheat and rice, may resonate like quarter-wave arials to their driving frequencies.

The results should transform agricultural practice. Once the process is understood, loudspeakers in fields and greenhouses will broadcast DREADCO's Green Music to the growing crop. Even by rock standards it will be a boringly monotonous blare. But the plants will love it. With its frequency tuned to vibrate their leaves in resonance, and its waveform matched to the rheology of their sap, it will spur them on to unprecedented growth and vigour.

Weeds, by contrast, could be selectively killed. Natural asymmetric waveforms, produced by impulsive turbulence or mechanical impact, have a sharp 'attack' followed by a gentler decay. But simply by playing a sound backward, modern electronics can easily reverse this state of affairs. The resulting unnatural waveforms should pump the sap back into the leaf, arresting its transpiration. Green Music will include a subtheme of inverted 'killer' tones, lethally tuned to the weeds and causing them to wilt and die. No nasty chemical herbicides will be needed. Ecologists and new-age mystics everywhere will rejoice.

David Jones

Mexican site for K/T impact crater?

SIR — A decade ago Penfield and Camargo¹ interpreted gravity and magnetic anomalies from northwestern Yucatan, Mexico, as evidence for a large, buried extraterrestrial impact crater. Research throughout the Caribbean^{2–6} suggests that this crater, now named the Chicxulub crater³, could be the site of the impact purported to have caused mass extinctions at the Cretaceous/Tertiary (K/T) boundary⁷. Using Landsat Thematic Mapper imagery of the Yucatan, we identified⁸ a semicircular ring of sink holes, known locally as cenotes, which correlates with the geophysical anomalies noted by Penfield and others^{1,3} (Fig. 1). We propose that the origin of the cenote ring is related to post-impact subsidence of the Chicxulub crater rim.

The cenote ring forms a nearly perfect semicircular boundary, 170 km in diameter between unfractured (within the ring) and

fractured Tertiary limestones, truncated by the coast and centred 17 km east of Progreso (Fig. 1). This boundary forms a barrier to lateral groundwater migration, causing

stresses along adjacent fault systems.

Evidence of subsidence is found in the negative gravity anomaly³ concentric with and just outside the ring (Fig. 1). Additional evidence of subsidence is the offset of Upper Cretaceous and earlier strata beneath the ring (Fig. 1), which may represent buried

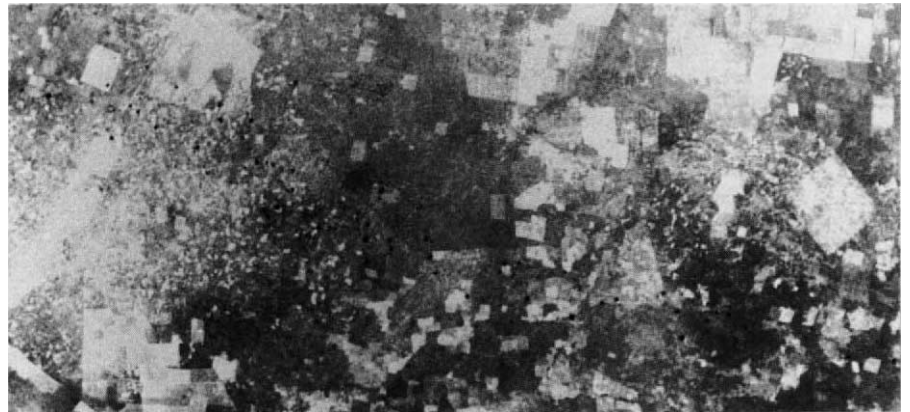


FIG. 2 Landsat Thematic Mapper band 5 (infrared) image of a portion of the cenote ring (location shown in Fig. 1). Note chain of cenotes (black dots) across the centre of the image, about 31 km. Landsat data from EOSAT Co., Lanham, Maryland, USA.

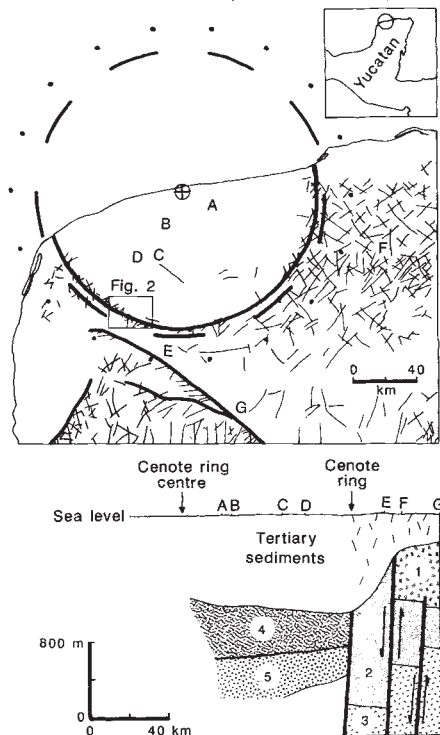


FIG. 1 Structural (upper) and subsurface (lower) geology of the cenote ring, northwestern Yucatan, Mexico (inset). Map fracture traces (thin lines) and faults (thick lines) from ref. 10. Semicircle, cenote ring; dashed circle, approximate location of negative gravity anomaly; dotted circle, approximate outer limit of concentric positive magnetic anomaly. Anomalies from Penfield and others^{1,3}. Subsurface data from drill holes are described by Weidie¹¹ and Lopez Ramos¹², and plotted as a function of the radial distance from the cenote ring centre (hole locations lettered on the map and across the top of the cross-section). Thick lines with arrows show subsidence along possible ring faults; thin lines show fracturing in Tertiary rocks. Key: (1) breccia (ejecta?); (2) Upper Cretaceous marine sediments; (3) Lower Cretaceous marine sediments; (4) breccia (impact?) and crater fill; (5) volcanic rock (impact melt?).

increased flows, dissolution and collapse along the boundary⁹. Large groundwater flows along the boundary are indicated by a valley-shaped depression in the groundwater surface centred on the ring, and by freshwater springs found where the coastline intersects the ring^{8,9}. The cenotes formed by the collapse process are 50–500 m diameter water bodies with depths of 2–120 m. Cenote density and width of the ring vary from about three cenotes per km² along a 3-km-wide portion in the southwest (Fig. 2), to a chain of single cenotes 3 km apart in the southeast. This variability is apparently related to differences in the flow of groundwater and fracturing outside the ring.

The fracturing that created the cenote ring was almost certainly caused by a circular structure, because no combination of linear stresses would be likely to produce such a nearly perfect circular feature. Except for the fractures, the Tertiary limestones are undeformed, suggesting that the fractures are related to a buried pre-Tertiary structure. A buried impact crater or volcanic caldera could produce a circular structure of this size. We discount the latter possibility because collapse of a caldera would cause fracturing within the ring, and volcanic rocks are found beneath the centre of the ring, not outside as would be expected for a caldera (Fig. 1).

On the other hand, post-impact subsidence induced by slumping and viscous relaxation in the rim of the proposed Chicxulub crater could well have caused the fracturing outside the cenote ring. The magnitude of this subsidence need not have been great to fracture the Tertiary limestones. Viscous relaxation may have been by only metres or tens of metres over the millions of years since the crater was buried. Craters this size have wide or multiple rims, but the fracturing beyond 40 km east and south of the ring is probably related to

ring faults typical of impact crater rims.

If there is indeed a crater, the region within the cenote ring corresponds to its floor; the crater rim diameter would then probably be >200 km. If confirmed as a site of impact, the Chicxulub crater would be the largest terrestrial impact crater known, which is consistent with the uniqueness of the Cretaceous/Tertiary global catastrophe.

KEVIN O. POPE

Geo Eco Arc Research,
2222 Foothill Boulevard, Suite E-272,
La Canada,
California 91011, USA

ADRIANA C. OCAMPO

Jet Propulsion Laboratory,
4800 Oak Grove Drive, MS 183–601,
Pasadena,
California 91109, USA

CHARLES E. DULLER

NASA Ames Research Center,
MS 242–4,
Moffett Field,
California 94035, USA

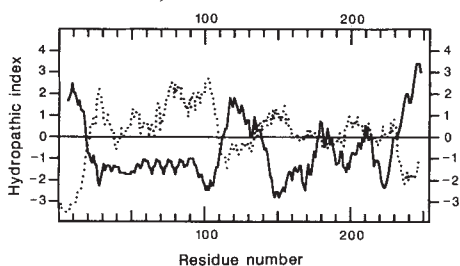
1. Penfield, G. T. & Camargo, Z. A. A. *Mtg Soc. Explor. Geophys. Abstr.* **51**, 37 (1981).
2. Hildebrand, A. R. & Boynton, W. V. *Science* **248**, 843–847 (1990).
3. Hildebrand, A. R. & Penfield, G. T. *Eos* **71**, 1425 (1990).
4. Hildebrand, A. R. & Boynton, W. V. *Eos* **71**, 1424–1425 (1990).
5. Smit, J. *Nature* **349**, 461–462 (1991).
6. Sigurdsson, H. et al. *Nature* **349**, 482–487 (1991).
7. Alvarez, L. W., Alvarez, W., Asaro, F. & Michel, H. V. *Science* **208**, 1095 (1980).
8. Pope, K. O. & C. E. Duller, in *III Simposio Latinoamericano Sobre Sensores Remotos, Memoria* (ed. Alvarez, R.) 91–98 (Sociedad de Especialistas Latinoamericanos en Percepcion Remota and Instituto de Geografia, UNAM, Mexico, 1989).
9. Marin, L. E. et al. in *Hydrology Papers from the 28th Int. Geol. Congr.* ed. Simpson, E. S. (Int. Assoc. Hydrol., in the press).
10. *Carta Geologica, 1:250,000* (Secretaria de Programacion y Presupuesto, Mexico, 1983).
11. Weidie, A. E. in *Geology and Hydrology of the Yucatan and Quaternary Geology of Northeastern Yucatan Peninsula* (eds Ward, W. C., Weidie, A. E. & Back, W.) 1–19 (New Orleans Geol. Soc., 1985).
12. Lopez Ramos, E. *Geologia de Mexico, Tomo III* (Mexico, 1979).

Anticipating the anti-prion protein?

SIR — A host-encoded prion protein (PrP) is crucial in the pathogenesis of transmissible spongiform encephalopathies, such as scrapie and scrapie-like diseases in man and animals¹. The PrP gene is highly conserved in evolution and contains a single open reading frame (ORF) located within a large exon. Several mutations in the human homologue PRNP gene were found in patients with familial Gerstmann-Sträussler-Scheinker's syndrome (GSS) and Creutzfeldt-Jakob disease (CJD).

While analysing PrP complementary DNA sequences with the DNA Strider computer program² I found a large overlapping ORF in the DNA strand opposite to the PrP transcriptional unit. The deduced amino-acid sequence of the prospective encoded protein was unique. Its hydropathy plot³ is almost a mirror image of that of the PrP (see figure). For simplicity the name of anti-PrP is used in reference to this putative protein.

This ORF may be of biological importance first, because it is as large as the PrP ORF. Second, none of the differences in PrP



Hydrophobicity plots³ of human PrP (solid line) and antiPrP (dotted line) proteins generated by the DNA Strider computer program². Numbers on the left and right indicate hydropathic index. Numbers above and below indicate amino acid positions in the PrP sequence.

sequences found in patients or animals resulted in additional stop codons in this ORF. It should be noted, however, that mutations found in GSS patients^{4,5} in codons 102 and 117 of the PRNP gene produced amino-acid changes in the anti-PrP, whereas mutations found in CJD patients^{6,7} in codon 178 and 200 did not. Third, there are ATG or CTG codons at the beginning of this ORF which could serve as translation initiation codons⁸. Fourth, existing data on PrP gene expression do not exclude expression of the anti-PrP gene because only double-stranded DNA probes were used to detect PrP messenger RNA¹. Obviously, only direct experimental analysis of infected and uninfected cells and tissues with single-stranded probes will show whether the anti-PrP gene is expressed.

The expression of the anti-PrP gene would introduce a new player, present a different view of scrapie infection, and raise new and important questions. Do PrP and anti-PrP proteins interact in infected cells⁹? Do GSS mutations, which, unlike CJD mutations, result in amino-acid changes in both proteins, explain the differences between the two

diseases? Do RNA unwindases¹⁰, capable of modifying double-stranded RNA substrates, produce changes in complementary PrP and anti-PrP mRNAs, thus altering the proteins?

In conclusion, the existence of a long anti-PrP ORF clearly warrants a search for the prospective encoded protein, especially in view of its potential role in transmissible spongiform encephalopathies.

D. GOLDGABER

Department of Psychiatry and
Behavioral Science,
State University of New York at Stony
Brook,
New York 11794-8101, USA

1. Prusiner, S. B. *Rev. Microbiol.* **43**, 345–374 (1989).
2. Marck, C. *Nucleic Acids Res.* **16**, 1829–1836 (1988).
3. Kyte, J. & Doolittle, R. F. *J. molec. Biol.* **157**, 105–132 (1982).
4. Hsiao, C. *et al. Science* **250**, 1587–1590 (1990).
5. Doh-ura, K. *et al. Biochem. biophys. Res. Commun.* **163**, 974–979 (1989).
6. Goldfarb, L. G. *et al. Lancet* **337**, 425 (1991).
7. Goldgaber, D. *et al. Expl. Neurol.* **106**, 204–206 (1990).
8. Prats, H. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 1836–1840 (1989).
9. Brentani, R. R. *J. theor. Biol.* **135**, 495–499 (1988).
10. Lamb, R. A. & Dreyfuss, G. *Nature* **337**, 19–20 (1989).

Perceiving depth

SIR — In his News and Views article, (*Nature* **349**, 365–366; 1991) B. J. Rogers comments that B. G. Cummings, E. B. Johnston and A. J. Parker show conclusively (*Nature* **349**, 441–413; 1991) that the human visual system is not able to use vertical disparities in computing three-dimensional shape. But there is more to three-dimensional perception than having two eyes and binocular stereopsis.

The advantages of binocular vision became apparent to me when I was temporarily blind in one eye. With depth perception lost, ordinary household tasks were no problem because I was in a familiar environment, but I did notice difficulty when hanging kitchen implements on hooks at arm's length. The solution was simple. With the object held out towards the hook it was only necessary to turn my head slightly to the side, displacing the apparent relative positions of object and hook, and, in effect, making one eye record the two images normally seen by two for depth to be assessed correctly so the utensils could be hung.

Since reading the two articles I have now checked by closing one eye and repeating the experiment but moving my head up and down slightly to give vertical displacement of the image. The method still works.

In most experiments on vision the visual system is held still. Fortunately, in real life it is not, and practical problems are much easier to solve.

M. LAZARIDES

Yeolmbridge House,
Nr Launceston,
Cornwall PL15 8TH, UK

Oceanic disjunctions

SIR — Long-distance separations (disjunctions) in the geographical distributions of animals and plants are not uncommon¹. Most result either from long-distance dispersal or from extinction, leaving widely separated relicts. Disjunct distributions between oceanic islands in different oceans are rarer and more surprising. Valdebenito *et al.*'s claim² to have found a new one in the disjunction of *Peperomia* (Dicotyledons, Piperaceae) is a little out; this disjunction was first identified by Skottsberg 45 years ago³. Groves⁴ lists *Peperomia berteriana tristanensis* as "possibly native" to the Tristan da Cunha archipelago in the Atlantic, so the morphological evidence¹ of its distinction from the Juan Fernandez populations in the Pacific is welcome.

Another biogeographic connection between the Tristan da Cunha archipelago and a Pacific archipelago has been known for some time. The subgenus *Trogloscapomyza* of *Scaptomyza* (Diptera, Drosophilidae) is found only on Nightingale Island in the Tristan archipelago and on several islands of the Hawaiian archipelago⁵. This disjunction was first noted by Hackman⁶, who thought it might be a relict distribution. The suggestion that it arose from long-distance dispersal by birds was first made in 1981 (ref. 7), and indeed such dispersal seems to have been important in the spread of *Scaptomyza* round the world from its origin in Hawaii⁸.

Other disjunct distributions are known exclusively across oceanic islands. For instance *Trochetiopsis* (Dicotyledons, Sterculiaceae) occurs only on St Helena in the Atlantic (two species) and its closest relative *Trochetia* only on Mauritius and Réunion Islands in the Indian Ocean (six species)^{9,10}.

MARK WILLIAMSON

Department of Biology,
University of York,
York YO1 5DD, UK

1. Pielou, E. C. *Biogeography* (Wiley, New York, 1979).
2. Valdebenito, H. A., Stuessy, T. F. & Crawford, D. J. *Nature* **347**, 549–550 (1990).
3. Skottsberg, C. *Acta horti Gotoburg* **16**, 251–288 (1946).
4. Groves, E. *Bull. Br. Mus. nat. Hist.* **8**, 333–420 (1981).
5. Hardy, E. *Insects of Hawaii* **12** (Diptera: Cyclorhapha) (University of Hawaii Press, Honolulu, 1965).
6. Hackman, W. *Acta zool. Fenn.* **97**, 3–73 (1959).
7. Williamson, M. *Island Populations* (Oxford University Press, 1981).
8. Williamson, M. *Biol. J. Linn. Soc.* **20**, 3–10 (1983).
9. Williamson, M. *Nature* **309**, 581 (1984).
10. Mabberley, D. J. *The Plant Book* (Cambridge University Press, 1987).

Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references. □

Politics and paradox

Fritz Stern

The Kaiser's Chemists. By Jeffrey Allan Johnson. University of North Carolina Press: 1990. Pp.279. \$39.95.

In the two decades before the First World War, Germany was a country in ascendancy; no state in Europe could match German power, a power and pre-eminence that extended to virtually all fields of national life. It was a dynamic, disciplined society, with a rapidly expanding economy and a scientific establishment that had won the admiration and the envy of the world. That it was also a country in which the modern sectors coexisted with anachronistic political forms is well known; it was a country marked by internal conflicts and anxieties about subversive forces at home and 'encircling' enemies abroad.

Wilhelm II embodied the paradox of his country: convinced of his divine right to rule, insecure and covetous of his people's love and admiration, he was also drawn to the modern — to all that was practical in science and technology. In the field of science, Germany gradually evolved what I would call a state-industrial-scientific complex. There was scientific genius in abundance in that Germany, but genius had to be nurtured; it demanded institutional support — and in turn much of that benefited German industry. The institutional history of German science — a subject that has only recently received the attention it deserves — is intrinsically fascinating and in many respects still pertinent to some of our quandaries today.

The Kaiser's Chemists by Jeffrey Allan Johnson is a curious contribution to this difficult genre. It is a study in two parts: of the failure of three eminent scientists to realize their plans, first advanced in 1905, for an Imperial Institute for Chemistry, and of the success some years later of various groups to establish the Kaiser-Wilhelm-Gesellschaft (KWG) that in turn established the Kaiser Wilhelm Institutes — the first two, opened in 1912, were designated institutes for chemistry and for physical chemistry and electrochemistry.

The failure of the initial effort has received little attention and Johnson's book is most useful in this part. In 1905, the great German chemist Emil Fischer — with the support of two distinguished colleagues, Walther Nernst and Wilhelm Ostwald — sought to mobilize German industry and Reich officialdom in order that an Imperial Institute for Chemistry be established — an analogue to the Imperial Institute for Physics and Technology, created in 1887, at the instigation of Werner Siemens. (See the important work by David Cahan, *An Institute for an Empire: The Physikalisches-Technische Reichsanstalt, 1871–1918*, Cambridge, 1989.) Fischer and his colleagues had ex-

perienced in their own careers the difficulties of pursuing basic research at their respective universities: the pressures of teaching were ever increasing, and the resistance of their humanistic colleagues to enlarging the costly scientific sector remained unrelenting. Moreover, Fischer was troubled that Germany's clear lead in organic chemistry was not maintained in the newer and more specialized fields, especially in physical

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Wilhelm II — eponymous Emperor.

chemistry, with its heavy installation costs. Johnson emphasizes that especially Fischer understood that science was both collaborative and competitive, internationalist and nationalist: the example of new institutions created in the United States — endowed by Carnegie and Rockefeller — was viewed with apprehension in Germany. The chemical industry supported the new plans, but the Imperial Government, somewhat disappointed in the work of the earlier institute, and in any case not directly responsible for

education, a realm reserved to the member states of the Reich, declined to cooperate.

The second half of Johnson's book deals with the more familiar story of the founding of the KWG. It was a different response to the same recognized need, that is, the establishment of institutions where leading scientists could pursue their research unfettered by teaching responsibilities. All manner of plans and promoters coalesced: at the end of his career the Prussian czar for university affairs, Friedrich Althoff, uncrowned, controversial but relentless in his pursuit of excellence, had hoped to establish a German Oxford on the royal domains in Dahlem — then still a suburb of Berlin. After his death in 1908, it was the liberal theologian and imperial favourite Adolf von Harnack who persuaded the Emperor to lend his name to the establishment of a society that in turn would sponsor semi-independent institutes for research. The Prussian government was miserly in its support, but helped to mobilize philanthropy; a carefully orchestrated 'solicitation' of wealthy individuals — bankers and industrialists — took place, with the tacit understanding that major donors would have a voice in the work of the society and would have the added satisfaction of knowing that they were contributing to a patriotic effort with an imperial nimbus. The campaign to raise sufficient funds would sound familiar to American campaign managers; in the German case, the government discreetly supplied tax information so that the wealthiest could be singled out. Among the wealthy there was a disproportionate number of Jews — and they contributed disproportionately. Membership in the KWG enhanced status and conferred dignity. In 1912, the Kaiser, himself convinced that science and technology were elements of national strength, opened the first two institutes; two years later, responding to the patriotic mood of the day, the director of the Institute for Physical Chemistry and Electrochemistry, Fritz Haber, put his institute at the service of Germany's war effort. In fact it was Haber and some colleagues who persuaded Germany's military leaders that science — through the synthetic production of raw materials that could no longer be imported because of the British blockade, and through the invention of new weapons, most notably and horribly, of poison gas — would prove indispensable to the war effort. Indeed the mobilization of scientific and technological talent allowed Germany to prevail for as long as it did.

Johnson's book is a revised dissertation, originally completed in 1979. It embodies research in German government and business archives; the single most valuable source, it would seem, has been the papers of Emil Fischer, deposited in Berkeley. He acknowledges his debt to two earlier scholars. Lothar Burchardt and Günter Wendel, a scholar in what used to be the German Democratic Republic, who could exploit and perhaps sequester some of the great archival

Mary Evans

material that was at his disposal. Today, Johnson's work on the founding of the KWG has been largely superseded by a comprehensive work on the KWG and the Max Planck Society, a work by many hands, including Johnson's (and mine): *Forschung im Spannungsfeld von Politik und Gesellschaft. Geschichte und Struktur der Kaiser-Wilhelm/Max Planck Gesellschaft*, edited by Rudolf Vierhaus and Bernhard von Brocke (Stuttgart, 1990).

Johnson's well-researched book is marred by his relentless wish to give it an all-embracing theoretical framework — hence the constant refrain that all that he details is evidence of Germany's "conservative modernization". On the one hand, there is nothing strikingly new about the thesis that nineteenth-century Germany assimilated modern institutions into an existing, largely authoritarian culture. But what other models for modernization did Johnson have in mind? Radical or revolutionary modernization? His efforts to ground this much-vaunted theory in historical facts, to link the institutional history of science to actual politics leads over and over again to the "fallacy of misplaced concreteness". One example — a central one — must suffice: it is true that Wilhelm II's prestige was damaged by the *Daily Telegraph* affair of 1908, but there is little if any evidence that this celebrated instance of his political ineptitude hastened his support of an enterprise that trusted advisors had submitted to him and that would have corresponded to his more rational impulses in any case. The author would have done better to make more plausible connections between particular facts and rely less on an abstruse and pretentious theoretical framework. It is also regrettable that a book about scientists — many of whom had a superb command of language — should make so little effort at literary distinction.

Johnson rightly emphasized the importance of American developments in German eyes. Scientists sensed the new rival, whereas many humanists lamented the possible intrusion. Johnson quotes from the great reactionary classicist, Ulrich von Wilamowitz-Moellendorf, who in defending the — unthreatened — position of the Prussian Academy of Sciences blames the Kaiser Wilhelm Institutes for having been largely financed by industry: "We cannot blame industry for that, but it is very American." In 1903, Fritz Haber had caught the modernizers' mood when he wrote after a visit to the United States: "The American [economic] challenge has become a common slogan, and Bismarck's sentence about the Germans who fear no one but God would seem in business circles gradually to be seriously amended: and a little the United States." *The Kaiser's Chemists* confirms that German ambivalence about America has a long and important history. □

Fritz Stern is at Fayerweather Hall, Columbia University, New York, New York 10027, USA.

Groping in the dark

Murray Stewart

Biophysical Electron Microscopy. Edited by P. W. Hawkes and U. Valdre. Academic: 1990. Pp. 517. £61.50, \$132.

MOST microscopists, especially biological ones, like doing it in the dark, and it is not clear how welcome turning on the lights would be. Groping in the dark is not without its attractions and rewards. But for those who have tired of some of the more mundane pleasures, or who are stimulated by a detailed knowledge of the intricacies of the subject, *Biophysical Electron Microscopy* should provide hours of instruction and even pleasure.

As is traditional in such manuals, it opens with a detailed description of the equipment and its function. It is often amazing to discover some of the misconceptions harboured in this area, even by those thought to be quite experienced, and so a thorough grounding is invaluable. Basic anatomy focuses on the column, but gives a frank and useful discussion of a number of exotic variations as well as the more straightforward methods used by most. Chapters on instrumentation, electron-specimen interactions, image formation and contrast provide a valuable introduction that is often not found in books directed towards a biological audience. High resolution is discussed in detail and gives an insight into the sorts of new information that can be obtained using electron crystallography, while also introducing many of the problems that remain in this specialized field.

A monumental chapter on image processing is probably unrivalled in the literature in terms of thoroughness and rigour. This chapter alone would make the book invaluable for workers interested in a more quantitative view of structural biology. Radiation damage is treated in considerable detail and identifies many of the problems that stem from this area. Low-temperature techniques are treated a little superficially, but do give some good pointers to more specialized sources. Finally, a number of accessory techniques for determining composition (X-ray microanalysis, electron energy-loss spectroscopy, cathodoluminescence) are described.

Although undoubtedly valuable, the rather mathematical emphasis of this book may sometimes make it rather demanding on most biologists. At times, the expectations of the intended audience in terms of matrix algebra or even calculus may be exceeded and some areas would be more valuable with a greater emphasis on explaining basic principles in simple terms rather than only as equations. My impression is that 'biophysicist' is interpreted as physicist looking at biology rather than as a biologist using physical

methods to look at the living world.

A major problem for many biologists has been that most books on electron microscopy deal with it from the perspective of materials science. Clearly, a book with a distinctly biological perspective would be extremely valuable. Consequently it is with some considerable sense of frustration that one finds that so many chapters of the present book deal mainly with specimens, examples and problems drawn from materials science. Electron-diffraction patterns from silicon single crystals or AuZn alloy or high-resolution lattice images of gold are of very limited usefulness to biologists. Such materials-science objects differ in a number of significant ways to biological material, most particularly in terms of radiation damage, contrast and the relative importance of dynamic effects. The reason that biologists do electron microscopy differently is far more related to the differences between specimens than to any lack of knowledge about the techniques concerned. In fact, the apparent lack of appreciation by some of the authors of the differences between biological and materials-science objects seriously limits the usefulness of this book. Although at least most of the background information is collated into one place (rather than scattered over several books on materials science), a real opportunity to discuss these points from a biological perspective has been lost.

The excitement of modern structural biology often seems lacking from this book. Macromolecular organization is one of the secrets of life and to most of its practitioners, biophysical electron microscopy is exciting because of the unique insights it can give into this area. The book is depressingly short of examples of the achievements of these techniques and where the subject is heading, and I doubt that a physicist would be enthusiastic about entering biology after reading it.

But notwithstanding these criticisms, *Biophysical Electron Microscopy* is a valuable background reference and will be an indispensable source book for anyone seriously interested in structural biology at anything other than the morphological level. Although it suffers from the usual problems of cohesion and emphasis common to most multi-author volumes, it does combine a very substantial amount of diverse and extremely useful fundamental information about electron microscopy and its underlying physical principles. It is not so much a question of whether it could have been done better, but rather that it is a very substantial achievement to have done it at all. It may well be that for some, in addition to telling them all they ever wanted to know about electron microscopy, it also tells them why perhaps they were quite correct to be afraid to ask. It may well not prevent biologists from groping in the dark, but they will at least have a better idea of how to do it. □

Murray Stewart is at the MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, UK.

No holds barred

Guy T. Emery

Science and Cultural Crisis: An Intellectual Biography of Percy Williams Bridgman (1882–1961). By Maila L. Walter. *Stanford University Press*: 1990. Pp.362. \$42.50.

PERCY Williams Bridgman (1882–1961) was an unusually productive and unusually perceptive physicist who made a strong impression on the scientific community. "Brilliant, intense and dedicated" were the adjectives with which his colleagues led off their biographical memoir. How to compress substances under very high physical pressure, and how matter behaved under such high pressure, was his experimental field.

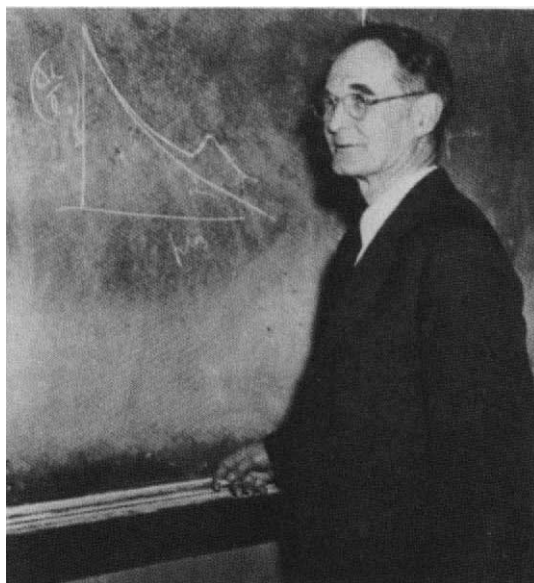
Maila L. Walter's *Science and Cultural Crisis* provides a detailed discussion of Bridgman's life and career. It is based on extensive work on Bridgman's papers and will reward the interest wide range of readers.

From the age of 18, Bridgman found a home at Harvard, as student, scholar and faculty member. As a graduate student he found a way to achieve hydrostatic pressures higher than previously possible. Leaking was eliminated by "a method of packing . . . which automatically becomes tighter the higher the pressure." He used his technical skill to measure several mechanical, electrical and thermal properties of a wide variety of materials over the new ranges of pressure made available, surmounting the challenging problems of calibration with ingenuity and perseverance. His work was careful and precise and was described in prose that, like his life, was straightforward and vigorous.

Approximately 200 scientific papers appeared between 1909 and 1958, later gathered in seven volumes by Harvard University Press. (*Collected Experimental Papers*, 1964). Many of the papers contain

extensive data on several different materials. The physics of materials under high pressure, as of the 1950s, was a field created by and dominated by Bridgman.

He worked alone, or with the help of a laboratory technician and a master machinist. The graduate students he supervised — never more than two at a time — pursued their own separate projects. He guarded his time for experimental work by negotiating his freedom from faculty committees, and taught, for most of his years, only graduate courses. In his modest rooms in the basements of Jefferson and Lyman Laboratories Bridgman laboured hard, with his mind and with his body, in pursuit of new data and new understanding.



Bridgman — "Brilliant, intense and dedicated".

The summers Bridgman spent in Randolph, New Hampshire, in a grand sloping valley in the White Mountains. He hiked, raised vegetables, read and played music with his family, and wrote up the accounts of his experiments. He also pondered and wrote about some of the deeper questions raised by physics and how physics is done. An examination of Tolman's attempt to exploit scale-invariance arguments led Bridgman into dimensional analysis, on which he published a book in 1922. (*Dimensional Analysis*, Yale University Press). A more general investigation of the 'meaning' of the conceptual terms used in physics, mostly stimulated by the special theory of relativity but with the interpretative puzzles of quantum mechanics also in mind, resulted in *The Logic of Modern Physics* (Macmillan, 1927), in which he proposed that "we mean by any concept nothing more than a set of operations; the concept is synonymous with the corresponding set of operations." (Bridgman's emphasis).

Bridgman's operational analysis struck a responsive chord among many scientists, and among quite a few philosophers as well. He was very much an individualist, however, and could not be subsumed even by the

Vienna Circle of philosophers, with whose approach his own had much in common. He continued to explore these deeper questions, in articles, and in books like *The Nature of Physical Theory* (Princeton University Press, 1936), *The Nature of Thermodynamics* (Harvard University Press, 1941), and *The Way Things Are* (Harvard University Press, 1959).

Some psychologists found operationalism particularly congenial, and through the efforts of S. S. Stevens applications and elaborations of Bridgman's line of thought played a considerable role for some time in that field.

If from Walter's book it seems to a non-philosophical reader that Bridgman's *sui generis* approach to deep questions is sometimes too much nibbled at for not conforming to comfortable philosophical classifications, there is recompense in the realization that his contributions are considered worth so much and so detailed an analysis.

Walter successfully covers the things Bridgman did that make him a culture hero to physicists. For example, she reminds us of his dictum that "The scientific method . . . is . . . doing one's damndest with one's mind, no holds barred" (*Reflections of a Physicist*, Philosophical Library, 1950); and of the manifesto once posted on his door: "I have decided from now on not to show my apparatus or discuss my experiments with the citizens of any totalitarian state." He did his best to maintain in himself and promote in others the highest standards of intellectual integrity.

Despite his rigour, he could be kind, for example to the young. In the strength and purity of his intellectualism he was not unlike Wittgenstein, with whom he shared a disquietude about the diagonal proof of non-denumerability. His operationism was in one sense a pointing at what scientists do, as more powerful than categorization by words.

In his later writings Bridgman stressed the privateness of science, and of human life itself. "Beyond the public level, waiting for a deeper analysis, is the private level. It is on the private level that I realize my essential isolation; here is my awful freedom that I can hardly face" (*Reflections*, p.75). Walter is good on this important existential strain in Bridgman.

Into his middle seventies Bridgman rode a bicycle to work. When faced by rapidly spreading inoperable cancer his response was as stoic, rational and straightforward as the rest of his life: he put the index to his collected scientific papers in the mail, wrote a brief note, and shot himself. Bridgman's life and his writings had a deep effect on physicists. Walter's book is a good reminder of this. □

Guy T. Emery is in the Department of Physics, Bowdoin College, Brunswick, Maine 04011, USA.

NEW JOURNALS ISSUE

This year, *Nature's* annual new journals review supplement will appear in the issue of 3 October. Publishers and learned societies are invited to submit journals for review, taking note of the following criteria:

- Journals that first appeared after June 1989 and issued at least four separate numbers by the end of April 1991 will be considered.
- The deadline for submission is end of May.
- Journals covering any aspect of science are eligible, but those dealing with clinical medicine, engineering and pure mathematics are excluded, as are publications of abstracts.
- The journals must be published at least three times a year.
- The main language used must be English.

When submitting journals for review, please send at least four different issues (the first, the most recent and any two others).

Cells evolving

T. Cavalier-Smith

Blueprint for a Cell: The Nature and Origin of Life. By C. de Duve. Patterson/Portland: 1991. Pp.275. \$19.95, £17.95.

For almost 3,000 million years living organisms were essentially unicellular: all evolution was cell evolution. But, curious though it may seem, much less has been written about the evolution of cells than about the relatively brief chemical evolution that preceded it or the recent evolution of multicellular organisms. De Duve's attractive and well-written book, *Blueprint for a Cell*, is therefore most welcome. The author is best known for 'discovering', or rather establishing the true nature of, lysosomes and peroxisomes. His earlier *A Guided Tour of the Living Cell* (Scientific American Books, 1984) was one of the best semipopular introductions to cell biology. The present book also deserves to be widely read.

Its first part outlines the most essential features of living organisms and how these are differently expressed in bacterial and eukaryotic cells. This leads to a reconstruction of the ancestral cell, in preparation for the second part which attempts to suggest how this ancestral cell may have originated. Part one is a rather well-balanced treatment that generally avoids excessive bias in one or other fashionable directions. But it does somewhat overemphasize the living state, at the expense of the processes of cell reproduction, which are absolutely central to an understanding of the dynamics of cell evolution.

The chapter on bacteria does not even mention the vital role of the rigid cell wall in cell growth and division and in providing a rigid framework for the segregation of DNA. Therefore the drastic implications of its loss for the origin of the eukaryotic cell are only partially appreciated. When discussing the origin of mitosis de Duve suffers from a widespread misconception, often propagated in even the best textbooks, that dinoflagellate chromosomes are not attached to spindle microtubules, and that segregation by membrane growth was a primitive eukaryote condition. In fact there is no reason to think that fluid lipid bilayers are able to segregate DNA in any organism. Some sort of skeleton, whether an exoskeleton as the bacterial wall or an endoskeleton as the eukaryote cytoskeleton, are essential for the reliable transmission of genetic information from generation to generation. The changeover from the bacterial to the eukaryotic pattern must have been far more traumatic than de Duve recognizes.

The whole book suffers from a gradualistic and a deterministic bias. The author does not recognize the evolutionary importance of historical contingency or of long periods of stasis followed by sudden change. Far too

little note is taken of the fossil record, which suggests that eukaryotes evolved only about half as long ago as bacteria (1.7×10^9 years ago, not $3.5\text{--}2.3 \times 10^9$ years ago as the author implies), and in no way supports the author's view that the origin of eukaryotes and of ourselves was inevitable. To dismiss chance and historical accidents as miracles and to allow only strict determinism is philosophically unsound.

Although the author has been over-influenced by uncalibrated gradualistic 'molecular-clock' ideas in his dating, he rightly realizes that the most recent common ancestor of all life cannot possibly have been a 'nebulous' progenote, but must have been a highly developed bacterial cell. He criticizes my view that this was eubacterial—not archaeobacterial—in its lipids because he thinks that acylester lipids could have been invented twice; yet he inconsistently invokes possible biophysical reasons to explain why in archaeobacteria a changeover from isoprenoid ether to acylester lipids never occurred. If it never occurred for such reasons in archaeobacteria, why postulate it twice independently in eukaryotes and eubacteria? I am tempted to conclude that de Duve's reluctance to accept that the ancestral cell was a eubacterium is because he prefers to suppose it was an acid-resistant, thermophilic sulphur-dependent archaeobacterium, because this appears to bolster his dubious theory for the prebiotic origin of a bioenergetic system based on thioesters.

In contrast to the first part of the book which is basically sound, part two is wildly speculative and fundamentally flawed in its two central theses. The most fundamental of these is the idea that chemical evolution produced a complete protometabolism of metabolic pathways catalysed by noninformed oligopeptides before the origin of replication and translation; moreover this protometabolism, which is incredibly held to have included essentially all the currently existing biosynthetic pathways and therefore all the raw materials for life, was preserved in some way during the changeover from uninformed to coded protein synthesis. Neither half of this central thesis is remotely plausible. Yet in one sense this general line of thinking ought to be pursued, and studied experimentally, because it must surely be the case that replication and translation originated, not in almost pure systems of the sort currently studied in the laboratory but in incredibly complex mixtures of molecules produced by the factors (mass action and crude prebiotic catalysts) that de Duve so lucidly describes. Even if, contrary to de Duve's hopeful assertions, this 'protometabolism' did not in fact give rise directly to modern metabolism it might well, as he argues, have provided an essential prerequisite for the origin of the constituents of the 'RNA world' or some yet more primitive self-replicating system.

De Duve's second thesis is that his protometabolism evolved a complete bioenergetic system that was initially based on thioesters,

and later evolved substrate-level phosphorylation, all before the origin of replication, translation or the first cell. He rejects the more conventional idea that bioenergetics started with pyrophosphate, because of the low concentration of phosphate at neutral pH in the presence of calcium. But because his thioester theory also would not work at neutral pH and low temperatures, he suggests that life began not at neutral pH but in hot acid (despite the instability of many key biogenic molecules under these conditions). But if one can postulate special environmental conditions for thioesters why not also for phosphates? De Duve does not explain why the low concentration of phosphate was not equally a problem for the postulated later origin of phosphorylation and nucleotides. His reasons for rejecting the widely discussed idea that the first bioenergetic system was a membrane-based photophosphorylation (not substrate-level phosphorylation) are equally unclear: perhaps it simply reflects the traditional biases of animal biochemists, which de Duve openly admits he shares.

In much of part two the selective forces that favour each postulated stage are very vague and inexplicit. For example, when discussing the origin of translation he says that it was not "the quality of the messages that counts", but of the ability to synthesize polypeptides. But though selection can directly improve replication regardless of its other phenotypic consequences this cannot be true of translation: there must be some specific benefit of the product to the system.

De Duve recognizes the importance of the association of early replicating molecules with some structure, for example a membrane, that can itself grow and divide and therefore be subject to 'true darwinian selection' but is very vague as to the specific benefits of such association: if the first bioenergetic system depended on membranes, as in the phototrophic theory, the advantages are by contrast quite obvious. He favours the inside-out cell/obcell of G. Blobel and myself as a precursor for true cells, but is very vague as to the properties of his postulated protocell: in one place he assumes it was porous and in another impermeable to protons.

Even though he touches on many of the most important considerations for understanding the origin of cells there is relatively poor integration in part two between them, which is a pity because the subject of cell evolution (as opposed to the more specialized and limited molecular evolution) should above all else be integrative one. Nonetheless, though to some degree falling between two stools (too technical for the intelligent layman; insufficiently detailed or rigorously argued for the specialist), *Blueprint for a Cell* will be stimulating reading for a broad scientific audience. □

T. Cavalier-Smith is in the Department of Botany, University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada.

Quantum optical tests of complementarity

Marlan O. Scully, Berthold-Georg Englert & Herbert Walther

Simultaneous observation of wave and particle behaviour is prohibited, usually by the position-momentum uncertainty relation. New detectors, constructed with the aid of modern quantum optics, provide a way around this obstacle in atom interferometers, and allow the investigation of other mechanisms that enforce complementarity.

COMPLEMENTARITY distinguishes the world of quantum phenomena from the realm of classical physics. The lion's share of the credit for teaching us to accept complementarity as a fact and for insisting that we have to learn to live with it belongs to Niels Bohr. In 1927, when he was reviewing the subject at Como¹ in a speech delivered in honour of Count Alessandro Volta (1745–1827), quantum theory as we know it today was still new, and all examples used to illustrate complementarity referred to the position (particle-like) and momentum (wave-like) attributes of a quantum mechanical object, be it a photon or a massive particle. This is the historical reason why complementarity is often superficially identified with the 'wave-particle duality of matter'.

Richard Feynman, discussing the two-slit experiment in his admirable introduction to quantum mechanics², notes that this wave-particle dual behaviour contains the basic mystery of quantum mechanics. In fact, he goes so far as to say: "In reality it contains the only mystery."

Complementarity, however, is a more general concept. We say that two observables are 'complementary' if precise knowledge of one of them implies that all possible outcomes of measuring the other one are equally probable. We may illustrate this by two extreme examples. (A more general discussion is given in ref. 3.) The first example consists of the position and momentum (along one direction) of a particle: if, say, the position is predetermined then the result of a momentum measurement cannot be predicted and all momentum values are equally probable (in a large range). The second extreme involves two orthogonal spin components of a spin-1/2 particle: if, say, the vertical spin component has a definite value ('up' or 'down') then upon measuring a horizontal component both values ('left' or 'right', for instance) are found, each with a probability of 50%.

Here then is the 'Principle of Complementarity':

For each degree of freedom the dynamical variables are a pair of complementary observables.

A less formal, less precise version in practical terms is:

No matter how the system is prepared, there is always a measurement whose outcome is utterly unpredictable.

Thus, in the microcosmos complete knowledge of the future in the sense of classical physics is not available. This, in essence, is the 'mystery' pointed to by Feynman.

As is true for all physical principles, the actual mechanisms that enforce complementarity vary from one experimental situation to another. Over the years various gedanken experiments have been analysed which emphasize this complementarity in quantum mechanics. Examples include Albert Einstein's recoiling-slit arrangement⁴ (analysed in the spirit of Willis Lamb⁵ in ref. 6), Feynman's electron-light scattering scheme² and Werner Heisenberg's microscope⁷. In the first two of these examples Heisenberg's position-momentum uncertainty relation⁸

$$\Delta x \Delta p \geq \frac{\hbar}{2} \quad (1)$$

makes it impossible to determine which hole the electron (or photon) passes through without at the same time disturbing the

electrons (photons) enough to destroy the interference pattern. Similar conclusions are reached by Heisenberg in his classic microscope example.⁷ In the present work we have found a way around this position-momentum uncertainty obstacle.

That is, we have found a way, based on matter-wave interferometry, and recent advances in quantum optics, namely the micromaser^{9–14} and laser cooling^{15,16}, to obtain which-path or particle-like information without scattering or otherwise introducing large uncontrolled phase factors into the interfering beams. To be sure, we find that the interference fringes disappear once we have which-path information, but we conclude that this disappearance originates in correlations between the measuring apparatus and the systems being observed. The principle of complementarity is manifest although the position-momentum uncertainty relation plays no role.

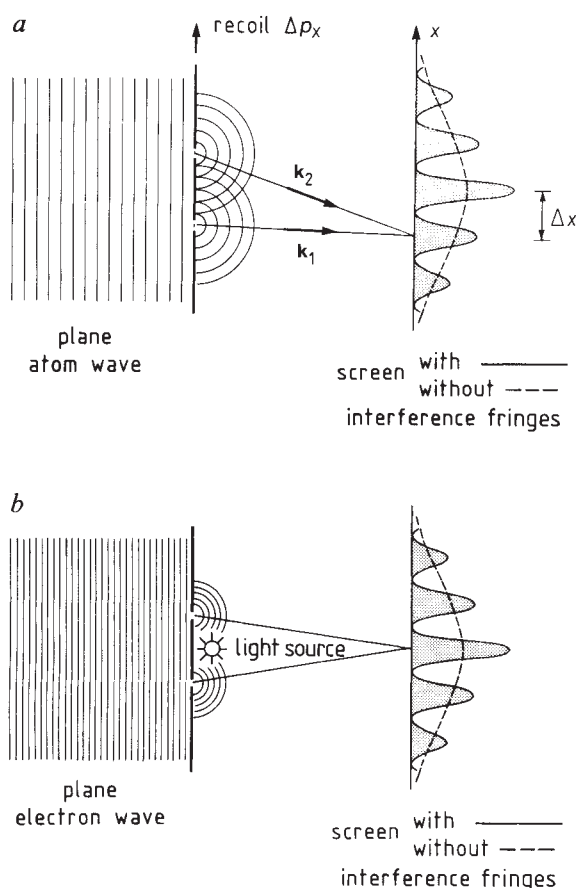


FIG. 1 *a*, Einstein's variant of the two-slit experiment. In this gedanken experiment the slits can recoil and reveal through which slit the photon reached the screen, inasmuch as only one of the wave vectors k_1 and k_2 is consistent with a known amount of recoil momentum. *b*, Feynman's version of Einstein's gedanken experiment. Here electrons interfere, and the scattering of photons is used to detect their position just behind the slits, revealing through which slit the electron reached the screen.

Gedanken experiments illustrating complementarity

We now turn to a brief survey of the usual textbook examples. (As a recent textbook we recommend ref. 17.) These examples traditionally involve a two-slit experiment in which light is allowed to interfere on a distant screen, thereby showing interference phenomena, the hallmark of wave-like behaviour. However, if we are able to detect which path the light has followed, we have particle-like information, and Nature refuses to let us observe wave-like phenomena.

Perhaps the archetypal example is Einstein's recoiling-slit arrangement^{4,6}, depicted in Fig. 1a. Einstein hoped, by this example, to give a gedanken experiment which would yield both which-path (German: *welcher Weg*) information and also show wave-like interference phenomena. But Bohr⁴ pointed out that we must also treat the recoiling slits by the laws of quantum mechanics. As discussed in Box 1, one then learns that there cannot be an interference pattern if the experiment allows us to determine through which slit the photon reached the screen.

In another example along these lines Feynman² replaces the photons by electrons. As the wave nature of matter is well known, interference between the electrons passing through slits, as in Fig. 1b, would be expected to lead to the usual fringe pattern on the screen. (Indeed, precision experiments, in which slow neutrons with a de Broglie wavelength of ~ 20 Å pass through two macroscopic slits with widths of ~ 150 μm demonstrate perfect agreement with the quantum mechanical predictions; see Fig. 2.) In this scheme we now have an extra 'handle' on the interfering particles as electrons can be observed by, for example, light scattering. This is depicted in Fig. 1b where we see a light source which would scatter light from the vicinity of either slit depending on which slit the electron comes through. Feynman then goes on to explain that this observation procedure destroys the interference pattern as seen on the screen. He concludes his analysis of this interesting example with the following statement:

If an apparatus is capable of determining which hole the electron goes through, it *cannot* be so delicate that it does not disturb the pattern in an essential way. No one has ever found (or even thought of) a way around the uncertainty principle.

In the experimental situations discussed so far, as in all standard examples, including Heisenberg's famous microscope⁷, complementarity is enforced with the aid of Heisenberg's position-momentum uncertainty relation. Is this mechanism always at work? No! We have recently¹⁸⁻²⁰ found a way around it.

We have developed and analysed a scheme that is very much in the spirit of Einstein's original proposal: we observe which path the particle has followed and do this without appreciably altering the spatial wave function. If we can do this, we will have shown that Einstein's goal is realizable, and the question

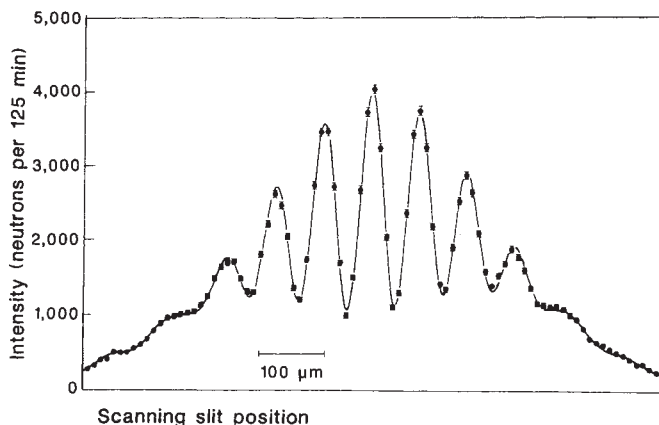


FIG. 2 Interference pattern produced by slow neutrons passing through a double slit. The solid curve represents the quantum mechanical prediction without any fitting. This plot is taken from ref. 34.

of how the principle of complementarity is enforced must then be readdressed. Here we will show that Einstein's goal is indeed obtainable: it is possible to obtain *welcher Weg* information without exposing the interfering beams to uncontrollable scattering events.

On the other hand, Bohr would not have been distressed by the outcome of these considerations, as wave-like (interference) phenomenon is lost as soon as one is able to tell which path the atom traversed. Quantum mechanics contains a built-in safeguard such that the loss of coherence in measurements on quantum systems can always be traced to correlations between the measuring apparatus and the system being observed. That is to say, in the present example, it is simply the information contained in a functioning measuring apparatus that changes the outcome of the experiment, and not uncontrollable alterations of the spatial wave function, resulting from the action of the measuring apparatus on the system under observation.

These considerations are based largely on recent advances in the field of quantum optics, in particular the development of micromaser techniques⁹⁻¹⁴. In these experiments one can ensure that an atom passing through a cavity will make a transition from an excited state to a lower state because of the interaction with the photons in the cavity.

To appreciate the logic of the present scheme, let us consider a beam of atoms replacing the light beam in the Einstein-Bohr dialogue, and the electron beam in the Feynman example. Just as in the previous cases, a beam of atoms incident upon a two-slit arrangement will show an interference pattern. As indicated in Fig. 3, a series of wider slits is used as collimators to define two atomic beams that arrive at the narrow slits where the interference pattern originates.

Let us disregard for the moment the laser and the maser cavities indicated in Fig. 3. In the interference region, the wave function describing the centre-of-mass motion of the atoms is then the sum of two terms referring to the two slits

$$\Psi(\mathbf{r}) = \frac{1}{\sqrt{2}} [\psi_1(\mathbf{r}) + \psi_2(\mathbf{r})] \quad (2)$$

and the probability density of particles falling on the screen where $\mathbf{r} = \mathbf{R}$, denoted by $P(\mathbf{R})$, will be given by the squared modulus of $\Psi(\mathbf{R})$, that is

$$P(\mathbf{R}) = \frac{1}{2} [|\psi_1(\mathbf{R})|^2 + |\psi_2(\mathbf{R})|^2 + \psi_1(\mathbf{R})^* \psi_2(\mathbf{R}) + \psi_2(\mathbf{R})^* \psi_1(\mathbf{R})] \quad (3)$$

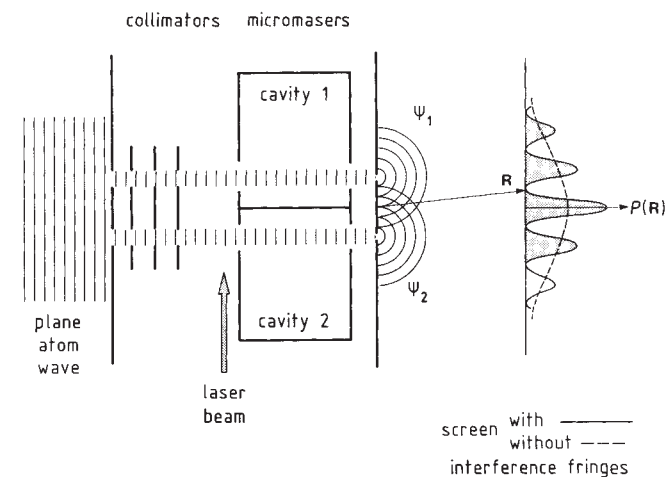


FIG. 3 Two-slit experiment with atoms. A set of wider slits collimates two atom beams which illuminate the narrow slits where the interference pattern originates. The collimation of the atomic beams would actually be done using atomic optics. One could, for instance, employ six-pole fields operating either on the magnetic dipole moment, or in the case of Rydberg atoms on the field-induced electric dipole moment. This set-up is supplemented by two high-quality micromaser cavities and a laser beam to provide which-path information.

We note that the usual interference behaviour is represented by the cross-terms $\psi_1^* \psi_2 + \psi_2^* \psi_1$.

Now just as Feynman's electron beam provided us with another handle on the interfering particles, not present in the case of interfering light beams, so, in the present case of an atomic system, we have further degrees of freedom involving the internal structure of the atom that are not available to us in the electron beam example.

In fact, we can now envisage preparing the atomic beam in an excited state (with the aid of a suitably operated laser) and then allowing the atoms to pass through the maser cavities in Fig. 3. On traversing either one of the cavities, the atom will emit a microwave photon and could leave *welcher Weg* information in the cavity.

One might think that the process of interacting with the microwave cavity fields and spontaneously emitting photons would disturb the centre-of-mass wave functions $\psi_1(\mathbf{r})$ and $\psi_2(\mathbf{r})$. The careful calculation reported in ref. 20 shows that this is not true. The natural way to discuss the centre-of-mass motion (the only essential parameter to be considered here) is in terms of kinetic and potential energy. In this language, the coupling between the atom and either one of the quantized cavity fields appears as a very small potential energy, whose sign and magnitude depends on the internal atomic and photonic quantum numbers. The wave function then consists of two components, one exposed to a weak attractive potential and the other to a repulsive one; the dynamical difference between attraction and repulsion effects the internal atomic transition accompanied by the emission of a photon. After the atom has traversed the cavity, it is again in force-free space and its momentum has the initial value. Thus, no net momentum is transferred to the atom during the interaction with the cavity fields. The de Broglie wave length of the atom is, therefore, not affected when a cavity photon is emitted, and so we have here an experiment which is "so delicate that it does not disturb" the interference pattern. (We should mention here that it is not possible to associate a definite momentum with a cavity mode, as this is not defined for a localized photon. Therefore the discussion of the atom-field interaction cannot be carried out on the basis of momentum transfer.)

In this sense we have conceived a *welcher Weg* detector which does not fall prey to the position-momentum uncertainty relation. How then are we to deal with the issue of complementarity? As discussed in the next section, it is simply the correlations between the detectors (micromaser cavities) and the atomic beams which are responsible for the loss of coherence (interference fringes) in the present experimental arrangement.

The above discussion of the *welcher Weg* detector was based on an atomic interference experiment. We should mention here that other experimental arrangements are also possible. For example, the two-field method developed by Norman Ramsey²¹ can be applied. If the two fields necessary for this method are produced in identical micromaser cavities which are being traversed by the atoms one after the other, then equation (3) applies as well. The quantum-beat experiment proposed in ref. 19 is another possible scheme.

In the next section the micromaser *welcher Weg* detector is studied in more detail. In the section after next, we then ask what happens if one erases the which-path information con-

tained in the *welcher Weg* micromaser cavities. Will interference fringes reappear?

A micromaser *welcher Weg* detector

A key ingredient in the micromaser *welcher Weg* detector is an excited atom which emits a photon when travelling through the cavity but not outside. (For a readable account of cavity electrodynamics, try ref. 22.) An atom in a long-lived Rydberg state, such as the $63p_{3/2}$ state of rubidium, is well suited to the present problem. In passing through the cavity it couples strongly either to $61d_{3/2}$ or to $61d_{5/2}$ at ~ 21 GHz as indicated in Fig. 4a. These states are currently used in micromaser experiments.

When such an atom is placed in a resonant cavity, it couples much more strongly to the microwave field and in fact decays rapidly from $63p_{3/2}$ (state *a*) to $61d_{5/2}$ (state *b*), for instance, because the mode density in the cavity (see Fig. 4b) is much larger than that in free space.

It is possible in principle, and realized in practice, for a Rydberg atom to make the transition $a \rightarrow b$ with unit probability when passing through the cavity, through spontaneous emission of a cavity microwave photon, even when the cavity does not contain photons initially.

Proceeding with the discussion of the micromaser *welcher Weg* detector, we return to Fig. 3 where, immediately preceding the masers, a laser beam is introduced, designed to excite all the atoms from the ground state to the excited state *a*. This can be accomplished by controlling the intensity of the laser beam such that this transition happens with certainty.

In the absence of the laser-cavities system, we now describe the atomic beam, after passing through the double slits, by the state vector

$$\Psi(\mathbf{r}) = \frac{1}{\sqrt{2}} [\psi_1(\mathbf{r}) + \psi_2(\mathbf{r})] |i\rangle \quad (4)$$

where $\mathbf{r}$ is the centre-of-mass coordinate and *i* denotes the internal state of the atom. Hence the probability density for particles on the screen at $\mathbf{r} = \mathbf{R}$ is given by the squared modulus of $\Psi(\mathbf{R})$,

$$P(\mathbf{R}) = \frac{1}{2} [|\psi_1|^2 + |\psi_2|^2 + (\psi_1^* \psi_2 + \psi_2^* \psi_1)] \langle i | i \rangle \quad (5)$$

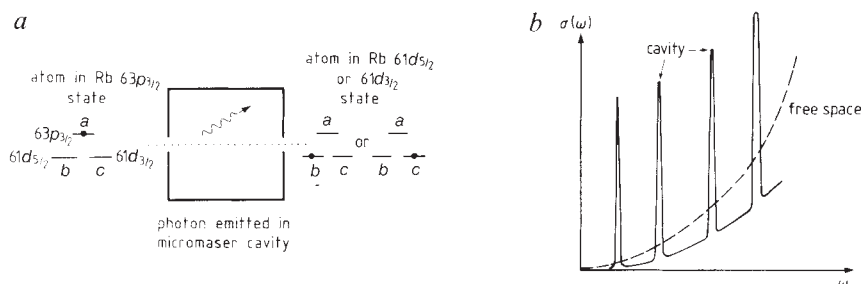
which, of course, agrees with equation (3).

Next consider the situation with the laser turned on and the ultracold (vacuum) micromaser cavities put into the two paths, as in Fig. 3. Before entering the cavities, the laser beam excites the atoms to the long-lived Rydberg state *a*. After passing through the cavities and making the transition $a \rightarrow b$, say, by spontaneous emission of a photon, the state of the correlated atomic beam and maser cavity system is given by

$$\Psi(\mathbf{r}) = \frac{1}{\sqrt{2}} [\psi_1(\mathbf{r}) |1_1 0_2\rangle + \psi_2(\mathbf{r}) |0_1 1_2\rangle] |b\rangle \quad (6)$$

where, for example, $1_1 0_2$ denotes the state in which there is one photon in cavity 1 and none in cavity 2. Please note that unlike (4) this $\Psi(\mathbf{r})$ is not a product of two factors, one referring to the atomic and the other to the photonic degrees of freedom. The system and the detector have become entangled by their

FIG. 4 a, Rubidium Rydberg atom in the $63p_{3/2}$ state *a* passes through a micromaser cavity, spontaneously emitting a microwave photon and making a transition to the $61d_{5/2}$ state *b* or the $61d_{3/2}$ state *c*. b, Density of photon modes, $\sigma(\omega)$, in free space (dashed curve) and in a micromaser cavity (solid curve), sketched as a function of the frequency ω .



interaction. In contrast to equation (3), the probability density at the screen is now given by

$$P(\mathbf{R}) = \frac{1}{2} [|\psi_1|^2 + |\psi_2|^2 + \psi_1^* \psi_2 \langle 1_1 0_2 | 0_1 1_2 \rangle + \psi_2^* \psi_1 \langle 0_1 1_2 | 1_1 0_2 \rangle] \langle b | b \rangle \quad (7)$$

But because $\langle 1_1 0_2 | 0_1 1_2 \rangle$ vanishes, the interference terms disappear here, so that

$$P(\mathbf{R}) = \frac{1}{2} [|\psi_1|^2 + |\psi_2|^2] \quad (8)$$

does not show fringes.

The micromasers will serve as *welcher Weg* detectors only if the one extra photon left by the atom changes the photon field in a detectable manner. Thus whether which-path information is available or not depends on the photon states initially prepared in the cavities. One extreme situation has just been discussed: no photons initially, one photon in one of the detectors finally. Clearly, here one can tell through which cavity, and therefore through which slit, the atom came to the screen. The situation is quite different when the cavities contain classical microwave radiation with large (average) numbers of photons, N_1 and N_2 , which have spreads given by their square roots. For instance, the change in photon number in cavity 1 is now from $N_1 \pm \sqrt{N_1}$ to $N_1 + 1 \pm \sqrt{N_1}$. This change cannot be detected, because $\sqrt{N_1} \gg 1$, so that there is no which-path information available. (For more details about *welcher Weg* detectors, consult ref. 23.)

In the latter situation (classical radiation in the micromaser cavities), we cannot tell through which slit the atom reached the screen and the interference pattern is just the same as in the absence of the micromaser cavities. In contrast, cavities containing no photons initially store which-path information and therefore the interference pattern is lost. It is changed to the incoherent superposition (8) of one-slit patterns.

We emphasize once more that the micromaser *welcher Weg* detectors are recoil-free; there is no significant change in the spatial wave function of the atoms. It is the correlation of the centre-of-mass wave function to the photon degrees of freedom in the cavities that is responsible for the loss of interference.

In this context, we point out related neutron^{24,25} experiments in which radio-frequency fields are employed to change the direction of the magnetic moment and thus the spin state: this does not affect the interference properties of the neutrons. These

experiments therefore demonstrate that we can indeed manipulate internal degrees of freedom without changing the centre-of-mass wave function of a quantum system.

Quantum eraser

In the preceding section we have seen that it is the system-detector correlations which account for the dramatic effects of the measuring apparatus on the system of interest. It is no surprise that coherence is destroyed as soon as one has *welcher Weg* information, but here no uncontrollable scattering events (as in Fig. 1a; see box) were involved in destroying the interference (wave-like) behaviour.

One then wonders whether it might not be possible to retrieve the coherent interference cross-terms by removing ('erasing') the *welcher Weg* information contained in the detectors. In this sense, we are here considering the quantum eraser problem as discussed sometime ago by M.O.S.²⁶ (inspired by John Wheeler's suggestion²⁷ of delayed-choice experiments) and also by others²⁸⁻³¹. If we erase the *welcher Weg* information in the microwave cavities, will spin coherence be restored? Notice that if we considered the coherence to be lost because of a random scattering or other stochastic perturbations, as studied in refs 32 and 33, for example, this question would never come up.

In fact, we shall see that interference effects can be restored by manipulating the *welcher Weg* detectors long after the atoms have passed. Edwin Jaynes²⁹ made some memorable remarks on this problem, which if adapted to the present context would read:

We have, then, the full EPR [Einstein-Podolsky-Rosen] paradox—and more. By applying or not applying the eraser mechanism before measuring the state of the microwave cavities we can, at will, force the atomic beam into either: (1) a state with a known path, and no possibility of interference effects in any subsequent measurement; (2) a state with both ψ_1 and ψ_2 present with a known relative phase. Interference effects are then not only observable, but predictable. And we can decide which to do after the interaction is over and the atom is far from the cavities, so there can be no thought of any physical influence on the atom's centre-of-mass wavefunction!

From this, it is pretty clear that present quantum theory not only does not use—it does not even dare to mention—the notion of a 'real physical situation'. Defenders of the theory say that this notion is philosophically naive, a throwback to outmoded ways of thinking, and that recognition of this constitutes deep new wisdom about the nature of human knowledge. I say that it constitutes a violent irrationality, that somewhere in this theory the distinction between reality and our knowledge of reality has become lost, and the result has more the character of medieval necromancy than of science. It has been my hope that quantum optics, with its vast new technological capability, might be able to provide the experimental clue that will show us to resolve these contradictions.

In the following we take up Jaynes' challenge, showing how to resolve this "paradox" and suggest further tests of complementarity in quantum mechanics within the framework of modern quantum optics. We present a gedanken experiment involving shutters and ideal photodetectors having unit quantum efficiency. This simple scheme is easy to understand and makes the physics clear, although it may not be possible to realize experimentally. Alternative schemes based on further application of the atomic beam(s)/micromaser combination, which we hope are more experimentally feasible, will be published elsewhere.

Consider now the arrangement of the atomic beam/micromaser system as indicated in Fig. 5a. There we see that the atoms pass through the two maser cavity detectors, but now we will imagine that the *welcher Weg* detectors are separated by a shutter-detector combination. So we now have a configuration in which the quantum eraser becomes possible. In particular, consider the cavity system in Fig. 5a. There we see two shutters arranged such that radiation will be constrained to remain either in the upper or the lower cavity, when the shutters are closed.

BOX 1 The Einstein-Bohr recoiling slit problem

Photons arriving on the (distant) screen of Fig. 1a at the location of the first side maximum of the fringe pattern, a distance Δx from the central maximum, possess different momenta $\hbar k_1$ or $\hbar k_2$ depending through which slit they reach the screen. The difference Δk_x of the x-component is well approximated by

$$\Delta k_x \approx \frac{2\pi}{\Delta x} \quad (a)$$

The recoil momentum of the plate supporting the slits must therefore be determined with a precision $\Delta p_x \sim \hbar \Delta k_x$, at least, to be able to tell through which slit the photon reached the screen. Thus $\hbar \Delta k_x$ must be distinctly larger than δp_x , the uncertainty in momentum of the slit plate, so that Heisenberg's uncertainty relation

$$\delta p_x \sim \frac{\hbar}{\delta x} \quad (b)$$

where δx is the uncertainty in position of the slit plate, implies

$$\Delta k_x > \frac{1}{\delta x} \quad (c)$$

In view of (a) this tells us that

$$\delta x \geq \Delta x \quad (d)$$

stating that the uncertainty in locating the slits (and therefore the fringes) is larger than the spacing between the fringes. In other words, the fringe pattern is washed out.

We further imagine that on opening the shutters, light will be allowed to interact with the photodetector wall. In this way the radiation, which is left either in the upper or in the lower cavity, depending upon whether the atom travelled along the upper or lower path, will now be absorbed and the 'memory of passage' (the *welcher Weg* information) could be said to be erased.

Do we now (after erasure) regain interference fringes? The answer is yes, but how can that be? The atoms are now far removed from the micromaser cavities and so "there can be no thought of any physical influence on the atom's centre-of-mass wave function". The answer to this question is given mathematically as follows.

Extending the mathematical description to include the detector, which is initially in its ground state d , we have

$$\Psi(\mathbf{r}) = \frac{1}{\sqrt{2}} [\psi_1(\mathbf{r})|1_0 0_2\rangle + \psi_2(\mathbf{r})|0_1 1_2\rangle] |b\rangle |d\rangle \quad (9)$$

which replaces equation (6). After absorbing a photon, the detector would be found in the excited state e .

It is now convenient to introduce symmetric, ψ_+ , and antisymmetric, ψ_- , atomic states defined as

$$\psi_{\pm}(\mathbf{r}) = \frac{1}{\sqrt{2}} [\psi_1(\mathbf{r}) \pm \psi_2(\mathbf{r})] \quad (10)$$

Likewise, we introduce symmetric, $|+\rangle$, and antisymmetric, $|-\rangle$, states of the radiation fields contained in the *welcher Weg* cavities,

$$|\pm\rangle = \frac{1}{\sqrt{2}} [|1_0 0_2\rangle \pm |0_1 1_2\rangle] \quad (11)$$

In terms of equations (10) and (11), the state (9) of the atom-beam/microwave-cavity/detector system appears as

$$\Psi(\mathbf{r}) = \frac{1}{\sqrt{2}} [\psi_+(\mathbf{r})|+\rangle + \psi_-(\mathbf{r})|-\rangle] |b\rangle |d\rangle \quad (12)$$

We now consider the interaction between the radiation field existing in the cavity and the detector. As mentioned earlier, we envisage the detector to consist of an atom with a lower state d and an excited state e . The interaction hamiltonian between field and detector depends on symmetric combinations of the

field variables, so that only the symmetric state $|+\rangle$ will couple to the fields.

We then find that the action of the detector (eraser) system produces the state

$$\Psi(\mathbf{r}) = \frac{1}{\sqrt{2}} [\psi_+(\mathbf{r})|0_1 0_2\rangle |e\rangle + \psi_-(\mathbf{r})|-\rangle |d\rangle] |b\rangle \quad (13)$$

That is, the symmetric interaction couples only to the symmetric radiation state $|+\rangle$; the antisymmetric state $|-\rangle$ remains unchanged.

Now, the atomic probability density at the screen goes as

$$P(\mathbf{R}) = \frac{1}{2} [\psi_+^*(\mathbf{R})\psi_+(\mathbf{R}) + \psi_-^*(\mathbf{R})\psi_-(\mathbf{R})] \\ = \frac{1}{2} [\psi_1^*(\mathbf{R})\psi_1(\mathbf{R}) + \psi_2^*(\mathbf{R})\psi_2(\mathbf{R})] \quad (14)$$

and does not show any interference fringes as long as the final state of the detector is unknown. But if one asks what is the probability density $P_e(\mathbf{R})$ for finding both the detector excited and the atom at $\mathbf{R}$ on the screen, the answer is

$$P_e(\mathbf{R}) = |\psi_+(\mathbf{R})|^2 \\ = \frac{1}{2} [|\psi_1(\mathbf{R})|^2 + |\psi_2(\mathbf{R})|^2] + \text{Re} [\psi_1^*(\mathbf{R})\psi_2(\mathbf{R})] \quad (15)$$

which exhibits the same fringes as equation (3), indicated as a solid line in Fig. 5b. In contrast, the probability density $P_d(\mathbf{R})$ for finding both the detector deexcited and the atom at $\mathbf{R}$ on the screen is

$$P_d(\mathbf{R}) = |\psi_-(\mathbf{R})|^2 \\ = \frac{1}{2} [|\psi_1(\mathbf{R})|^2 + |\psi_2(\mathbf{R})|^2] - \text{Re} [\psi_1^*(\mathbf{R})\psi_2(\mathbf{R})] \quad (16)$$

giving rise to the antifrings indicated by the broken line in Fig. 5b. If the eraser photon signal is disregarded, one obtains the superposition (14), equal to half the sum of P_e and P_d , which is fringeless, and, of course, identical with (8).

Here is the physical interpretation of this calculation. After an atom has run the gauntlet from the oven to the screen, passing through micromasers and leaving its tell-tale photon, we record an event somewhere on the screen. Then we return to the *welcher Weg* micromasers, open the shutters and allow the absorption of the microwave photon. When we observe a photocount in the detector we know that erasure has been completed. In this event the atom is counted as a 'yes'-atom.

Then we wait for another atom to pass through the system from oven to screen. Again we record an event on the screen and then turn to the micromaser cavities. This time suppose that, upon opening the shutter, we observe no photocount in the quantum eraser detector. This will be the case half of the time, as explained above. Now we count the atom as a 'no'-atom.

We repeat the above sequence many times. Eventually, the 'yes'-atoms will build up the solid-line fringes in Fig. 5b, and the 'no'-atoms produce the broken-line antifrings. Finally we note that the fringes and antifrings will cancel if we do not correlate them to the state of the eraser-detector. In this way we have resolved the 'Jaynes paradox'.

Having presented the physics of quantum erasure we now turn to an experimentally more realizable scheme which has much in common with the quantum eraser idea. We consider, as in Fig. 6, the asymmetric situation in which cavity 1 is tuned to the transition $a \rightarrow b$ ($63p_{3/2} \rightarrow 61d_{3/2}$), and cavity 2 is tuned to the transition $a \rightarrow c$ ($63p_{3/2} \rightarrow 61d_{5/2}$).

Even if the cavities contain classical microwave radiation, as we shall assume in the sequel, and therefore do not store which-path information, the screen will not show interference fringes because the internal atomic states b and c are orthogonal. This is analogous to the disappearance of the interference terms in equation (7), except that now the atoms themselves carry the *welcher Weg* information.

The latter circumstance again invites the question: could one not induce the transitions $b \rightarrow c$ in the atoms that traversed

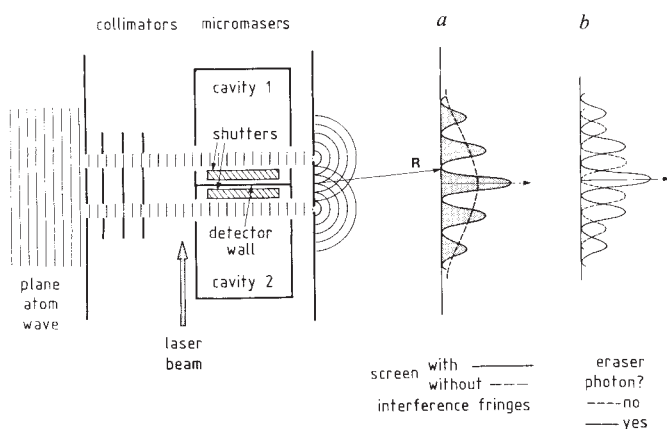


FIG. 5 a, Quantum erasure configuration in which electro-optic shutters separate microwave photons in two cavities from the thin-film semiconductor (detector wall) which absorbs microwave photons and acts as a photo-detector. b, Density of particles on the screen depending upon whether a photocount is observed in the detector wall ('yes') or not ('no'), demonstrating that correlations between the event on the screen and the eraser photocount are necessary to retrieve the interference pattern.

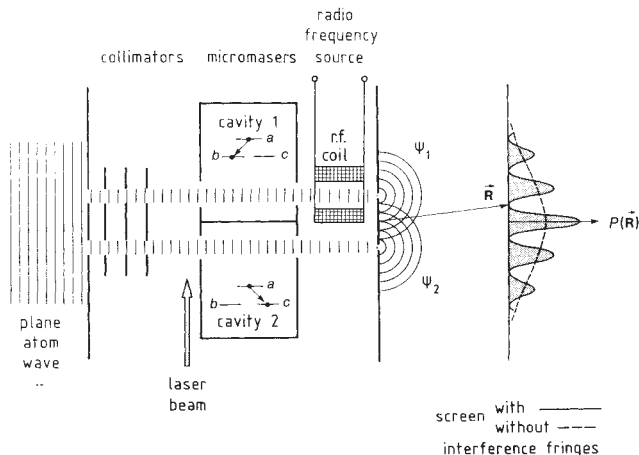


FIG. 6 Asymmetric set-up in which cavity 1 induces the transition $a \rightarrow b$ and cavity 2 induces $a \rightarrow c$. Which-path information is erased by the radio frequency in the coil where $b \rightarrow c$ happens.

cavity 1, so that the which-path information is erased, and thereby make the interference pattern reappear? The answer is affirmative. The actual experimental realization, however, is a delicate matter, because one must exert careful control on the phases of the various classical radiation fields. To appreciate what is involved, suppose that between cavity 1 and the slit plate there is a coil that can be fed with radio frequency of ~ 50 MHz with the right strength to ensure the transition $b \rightarrow c$, as depicted in Fig. 6. In the interference region the state of the atom is essentially

$$\Psi(\mathbf{r}) = \frac{1}{\sqrt{2}} [\psi_1(\mathbf{r}) + e^{i\beta} \psi_2(\mathbf{r})] |c\rangle \quad (17)$$

where the relative phase angle β is determined by the phases of the microwave fields in the two maser cavities and the radio-wave field in the coil. As these fields have different frequencies, β really refers to a certain instant, the moment, say, when the atom is excited to state a by the laser beam. The probability density at the screen

$$P(\mathbf{R}) = |\Psi(\mathbf{R})|^2 = \frac{1}{2} (|\psi_1|^2 + |\psi_2|^2) + \text{Re} (\psi_1^* e^{i\beta} \psi_2) \quad (18)$$

now exhibits an interference term that depends on β very sensitively. If, therefore, the value of β varies from atom to atom, the interference pattern will not build up. This illustrates quite

well the omnipresent phenomenon of coherence loss caused by random phases. Consequently, one must ensure that the phase angle β is the same for all atoms to make the interference fringes reappear. In the set-up of Fig. 6 this can be achieved by adjusting the phase of the radiofrequency radiation in the coil to the phases that the microwave fields in the cavities have at the moment when the laser excites the atom. An additional bonus is the possibility of varying the chosen value of β , which enables one to shift the interference pattern on the screen. In summary, the control over the phase angle β represents a switch with which the experimenter can turn the interference fringes on and off, or relocate them.

Thus it would seem that the way is open for experiments of the *welcher Weg*/quantum-eraser type. No doubt they will be difficult, but as we gain more experience with these 'amazing masers', experiments along these lines will one day be realized, and *welcher-Weg*-type experiments are now under way at the Max-Planck-Institut in Garching. $\square$

Marian O. Scully is at the Center for Advanced Studies and Physics and Astronomy Department, University of New Mexico, Albuquerque, New Mexico 87131, USA, and at the Max-Planck-Institut für Quantenoptik, D-8046 Garching bei München, Germany. Berthold-Georg Englert and Herbert Walther are at the Max-Planck-Institut für Quantenoptik and the Sektion Physik, Universität München, 8046 Garching, Germany. M.O.S. is supported by the Office of Naval Research. We dedicate this paper to Professor McAllister Hull.

1. Bohr, N. *Naturwissenschaften* **16**, 245–257 (1928).
2. Feynman, R., Leighton, R. & Sands, M. *The Feynman Lectures on Physics* Vol. III (Addison Wesley, Reading, 1965).
3. Schwinger, J. *Proc. natn. Acad. Sci. U.S.A.* **46**, 570–579 (1960).
4. Bohr, N. in *Albert Einstein: Philosopher–Scientist* (ed. Schilpp, P.A.) 200–241 (Library of Living Philosophers, Evanston, 1949); reprinted in *Quantum Theory and Measurement* (eds Wheeler, J. A. & Zurek, W. H.) 9–49 (Princeton University Press, 1983).
5. Lamb, W. E. Jr *Physics Today* **22**, 23–28 (April 1969).
6. Wothers, W. & Zurek, W. H. *Phys. Rev. D* **19**, 473–484 (1979).
7. Heisenberg, W. *The Physical Principles of the Quantum Theory* (University of Chicago Press, 1930).
8. Heisenberg, W. *Z. Phys.* **43**, 172–198 (1927).
9. Meschede, D., Walther, H. & Müller, G. *Phys. Rev. Lett.* **54**, 551–554 (1985).
10. Rempe, G., Walther, H. & Klein, N. *Phys. Rev. Lett.* **58**, 353–356 (1987).
11. Diedrich, F., Krause, J., Rempe, G., Scully, M. O. & Walther, H. *IEEE J. Quant. Electr.* **24**, 1314–1319 (1988).
12. Rempe, G., Schmidt-Kaler, F. & Walther, H. *Phys. Rev. Lett.* **64**, 2783–2786 (1990).
13. Haroche, S. & Raimond, J. M. in *Advances in Atomic and Molecular Physics* Vol. 20 (eds Bates, D. & Bederson, B.) 347–411 (Academic, Orlando, 1985).
14. Gallas, J. A. C., Leuchs, G., Walther, H. & Figger, H. in *Advances in Atomic and Molecular Physics* Vol. 20 (eds Bates, D. & Bederson, B.) 413–466 (Academic, Orlando, 1985).
15. Phillips, W. & Metcalf, H. *Scient. Am.* **256**, 36–42 (March 1987).
16. *Laser Cooling and Trapping of Atoms* (eds Chu, S. & Wieman, C.) *J. opt. Soc. Am. B* **6**, 2019–2278 (1989).
17. Cohen-Tannoudji, C., Diu, B. & Laloë, F. *Quantum Mechanics* (Wiley, New York, 1977).
18. Scully, M. O., Englert, B.-G. & Schwinger, J. *Phys. Rev. A* **40**, 1775–1784 (1989).
19. Scully, M. O. & Walther, H. *Phys. Rev. A* **39**, 5229–5236 (1989).
20. Englert, B.-G., Schwinger, J. & Scully, M. O. in *New Frontiers in Quantum Electrodynamics and Quantum Optics* (ed. Barut, A. O.) 513–519 (Plenum, New York, 1990).
21. Ramsey, N. F. *Molecular Beams* (Clarendon, Oxford, 1956).
22. Haroche, S. & Kleppner, D. *Physics Today* **42**, 24–30 (January 1989).
23. Englert, B.-G. & Scully, M. O. in *New Frontiers in Quantum Electrodynamics and Quantum Optics* (ed. Barut, A. O.) 507–512 (Plenum, New York, 1990).
24. Rauch, et al. *Phys. Lett. A* **54**, 425–427 (1975).
25. Badurek, G., Rauch, H. & Tuppinger, D. *Phys. Rev. A* **34**, 2600–2608 (1986).
26. Scully, M. O. & Drühl, K. *Phys. Rev. A* **25**, 2208–2213 (1982).
27. Wheeler, J. A. in *Mathematical Foundations of Quantum Theory* (ed. Marlow, A. R.) 9–48 (Academic, New York, 1978).
28. Greenberger, D. M. & Ya'sin, A. in *New Techniques and Ideas in Quantum Measurement Theory* (ed. Greenberger, D. M.) *Ann. N.Y. Acad. Sci.* **480**, 449–457 (1986).
29. Jaynes, E. in *Foundations of Radiation Theory and Quantum Electrodynamics* (ed. Barut, A. O.) 37–43 (Plenum, New York, 1980).
30. Peres, A. *Phys. Rev. D* **22**, 879–883 (1980).
31. Zajonc, A. G. in *Coherence in Quantum Optics* (eds Mandel, L. & Wolf, E.) 323–329 (Plenum, New York, 1984).
32. Prosperi, G. M. & Scotti, A. *J. Math. Phys.* **1**, 218–221 (1960).
33. O'Connell, R. F., Savage, C. M. & Walls, D. F. in *New Techniques and Ideas in Quantum Measurement Theory* (ed. Greenberger, D. M.) *Ann. N.Y. Acad. Sci.* **480**, 267–274 (1986).
34. Zeilinger, A., Gähler, R. & Skull, C. G. *Rev. mod. Phys.* **60**, 1067–1073 (1988).

Male development of chromosomally female mice transgenic for *Sry*

Peter Koopman, John Gubbay, Nigel Vivian, Peter Goodfellow* & Robin Lovell-Badge†

Laboratory of Eukaryotic Molecular Genetics, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

* Human Molecular Genetics Laboratory, Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK

† To whom correspondence should be addressed

The initiation of male development in mammals requires one or more genes on the Y chromosome. A recently isolated gene, termed *SRY* in humans and *Sry* in mouse, has many of the genetic and biological properties expected of a Y-located testis-determining gene. It is now shown that *Sry* on a 14-kilobase genomic DNA fragment is sufficient to induce testis differentiation and subsequent male development when introduced into chromosomally female mouse embryos.

THE processes of morphogenesis and cellular differentiation are dependent on the activity of sets of genes in complex interacting networks or pathways. Within these pathways, specific genes may be rate-limiting or act as a switch, such that their activity seems to cause a developmental event. One case where this occurs is in sex determination, where the pathways of gene activity that lead to male or female development must include a switch mechanism to determine which pathway is chosen^{1,2}. In mammals, the Y chromosome acts as a dominant male determinant³⁻⁵ as it carries a gene or genes with a critical role in the male pathway. The central event in mammalian sex determination is the differentiation of testes rather than ovaries from the indifferent gonad (genital ridge)^{6,7}. All other differences between the sexes in eutherian mammals are secondary effects due to hormones or factors produced by the gonads. For this reason sex determination is equivalent to testis determination, and the Y-chromosomal gene(s) responsible has been named *Tdy* (testis-determining gene on the Y) in mice, and *TDF* (testis-determining factor) in humans.

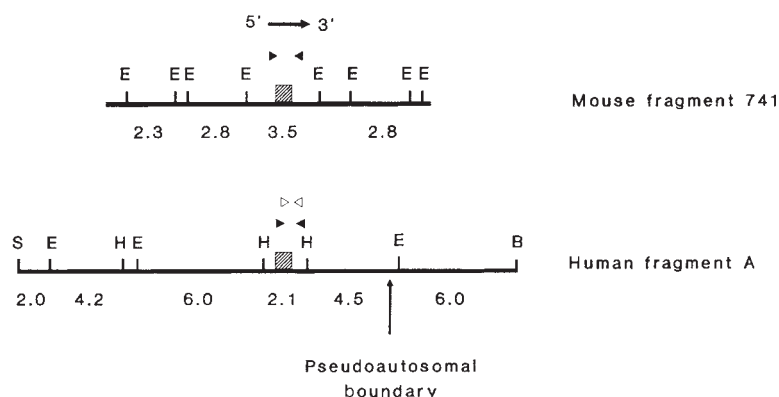
By exploiting detailed maps of the sex-determining region of the human Y chromosome, we cloned the gene *SRY* and its mouse equivalent *Sry*^{8,9}. These genes are located in the smallest regions of the human and mouse Y chromosomes known to be male-determining, and conserved homologues have been found on the Y chromosomes of all other eutherian mammals tested. Furthermore, *Sry* is deleted from a mouse Y chromosome with a mutant *Tdy*⁹⁻¹¹. As might be predicted for a regulatory gene, *SRY/Sry* encodes a protein containing a DNA-binding motif^{8,9}. *Sry* also shows a pattern of expression in the mouse entirely consistent with a role in testis determination, being expressed for a short period from about 10.5-12 days *post coitum* (d.p.c.) just before overt testis differentiation, specifically in the somatic cells of the genital ridge¹².

Direct evidence that *SRY/Sry* has a role in testis formation was obtained from the analysis of the genomes of XY females with gonadal dysgenesis. In two cases, sex-reversed XY daughters were found to have mutations in *SRY* that were not present in their fathers^{13,14}. This correlation between *de novo* mutation in *SRY* and sex reversal implies that *SRY/Sry* is required for normal testis formation, but the experiments do not address whether it alone is equivalent to the genetically defined factor *TDF/Tdy*.

The best way to test the function of *SRY/Sry* is to introduce it into XX embryos, and to see if they develop as males. The pattern of *Sry* expression during fetal gonad development in the mouse suggests that precise regulation of the gene may be critical for its action¹². We therefore introduced murine *Sry* or human *SRY* as part of a genomic fragment, in the expectation that this would provide the correct regulatory sequences. Although the human gene does not seem to function in mice, mouse *Sry* in a 14-kilobase (kb) genomic fragment gives rise to normal testis development in chromosomally female transgenic mice, as can be seen at both embryonic and adult stages.

FIG. 1 Genomic DNA fragments used for microinjection. Restriction maps of mouse *Sry* fragment 741 (ref. 9) and human *SRY* fragment A (isolated from the cosmid cAMF; ref. 29). Restriction-endonuclease sites: B, *Bam*HI; E, *Eco*RI; H, *Hind*III and S, *Sal*I. Fragment sizes are indicated in kb. The conserved *Sry/SRY* open reading frame is indicated by a shaded box. The direction of the open reading frame is shown above the two clones. The position of the human pseudoautosomal boundary is indicated, the pseudoautosomal region being to the right of this point. The positions of oligonucleotide primers used for PCR analysis (described in the legends to Figs 3 and 5) are indicated by triangles.

METHODS. *SRY-* or *Sry*-containing fragments were released from cosmid or phage vectors by digestion with appropriate restriction enzymes and then isolated by agarose gel electrophoresis and further purified by one of three methods: (1) GeneClean (Bio101), according to manufacturer's instructions; (2) phenol extraction, Sephadex G50 column chromatography and ethanol precipitation; (3) GeneClean followed by elutip (Schleicher & Schuell) and ethanol precipitation. Transgenic mice were produced essentially as described by Hogan *et al.*³⁸. In brief, three- to five-week-old (CBA $\times$ C57BL/10) F₁ females were superovulated and mated to F₁ stud males. The next day,



fertilized eggs were collected from oviducts of females showing vaginal plugs. Pronuclei were microinjected with 1-2 pl of DNA at a concentration of approximately 2 μ g ml⁻¹. The eggs were cultured overnight in M16 or T6 medium, and 2-cell embryos implanted by oviduct transfer into day-of-plug pseudopregnant recipients.

Sex reversal of transgenic mouse embryos

Fertilized eggs were microinjected with *Sry* gene sequences and transferred to pseudopregnant recipients, and some of the resulting embryos were analysed 14 days later rather than allowing them all to develop to term. The first visible sign of testis development from the genital ridge is the formation of testis cords at about 12.5 dpc in the mouse. This is due to the differentiation of Sertoli cells and their alignment into epithelial structures surrounding the germ cells¹⁵. Cord formation confers a characteristic striped appearance to the developing testis, distinguishing it from the fetal ovary. Other morphological changes characteristic of the testis are its rapid growth and prominent vasculature. Phenotypic sex can be rapidly assayed by examining fetuses about 14 days after oviduct transfer, when testis-cord formation is obvious even with partial sex reversal^{16,17}. An indication of chromosomal sex was obtained by staining for sex chromatin in amnion cells¹⁸. Where necessary, this was confirmed by Southern blot analysis or the polymerase chain reaction (PCR) using a DNA probe or oligonucleotide primers derived from the Y-linked gene *Zfy-1* (refs 19–21).

A 14-kb fragment derived from a phage clone L741 (ref. 9), containing about 8 kb of sequence upstream and 5 kb downstream, of the putative DNA-binding domain of *Sry* (Fig. 1), was purified from vector sequences and used to generate transgenic mice. After injection with this fragment (subsequently referred to as f741), 158 embryos were obtained. Most of these were XY males or XX females, in roughly equal proportion (Table 1). However, in two cases testes were seen in embryos whose sex chromatin indicated an XX rather than an XY sex chromosome constitution. Southern-blot analysis showed that

TABLE 1 Analysis of mouse fragment 741 transgenic embryos

No. of embryos	Sex chromatin	<i>Sry</i>	<i>Zfy</i>	Deduced karyotype	Transgenic	Phenotypic sex
63	+	—	27-/40 ND	XX	—	♀
27	—	+	+	XY	ND	♂
58	—	ND	ND	XY	ND	♂
2	—	—	—	XO	—	♀
6	+	+	—	XX	+ *	♀
2	+	+	—	XX	+	♂

Injected embryos were examined 14 days after transfer. Embryos were analysed in the following way: chromosomal sex (XX or XY/XO) was determined by staining for sex chromatin in amnion cells¹⁸. Transgenesis was assayed either by Southern blot or PCR detection of *Sry*, and the presence or absence of a Y chromosome judged from similar assays for *Zfy* gene sequences. Phenotypic sex was determined by scoring for testis or ovary development. The frequency of XO progeny was consistent with previous studies³⁵. Asterisk indicates that in four cases of XX transgenesis, comparison of the *Sry* signal to that of a control male indicated mosaicism for the transgene. The bold type highlights the transgenic embryos obtained. ND, not determined. Genomic DNA for Southern analysis was prepared from limbs essentially as described³⁶. For PCR analysis proteinase K digestion was performed in 1 mM EDTA, the DNA was extracted once with phenol/chloroform and a small aliquot added directly to the PCR reaction mixture. Southern analysis of *EcoRI*-digested DNA was performed as described by Maniatis *et al.*³⁷. For *Sry*, blots were probed with clone 422.04 (see ref. 9) which contains the *Sry* conserved motif. For the *Zfy* genes, a 1.9-kb *HindIII* genomic fragment containing the region encoding the *Zfy-1* zinc-finger domain^{11,21} was used as a probe. PCR analysis was performed as described in Fig. 3 legend.

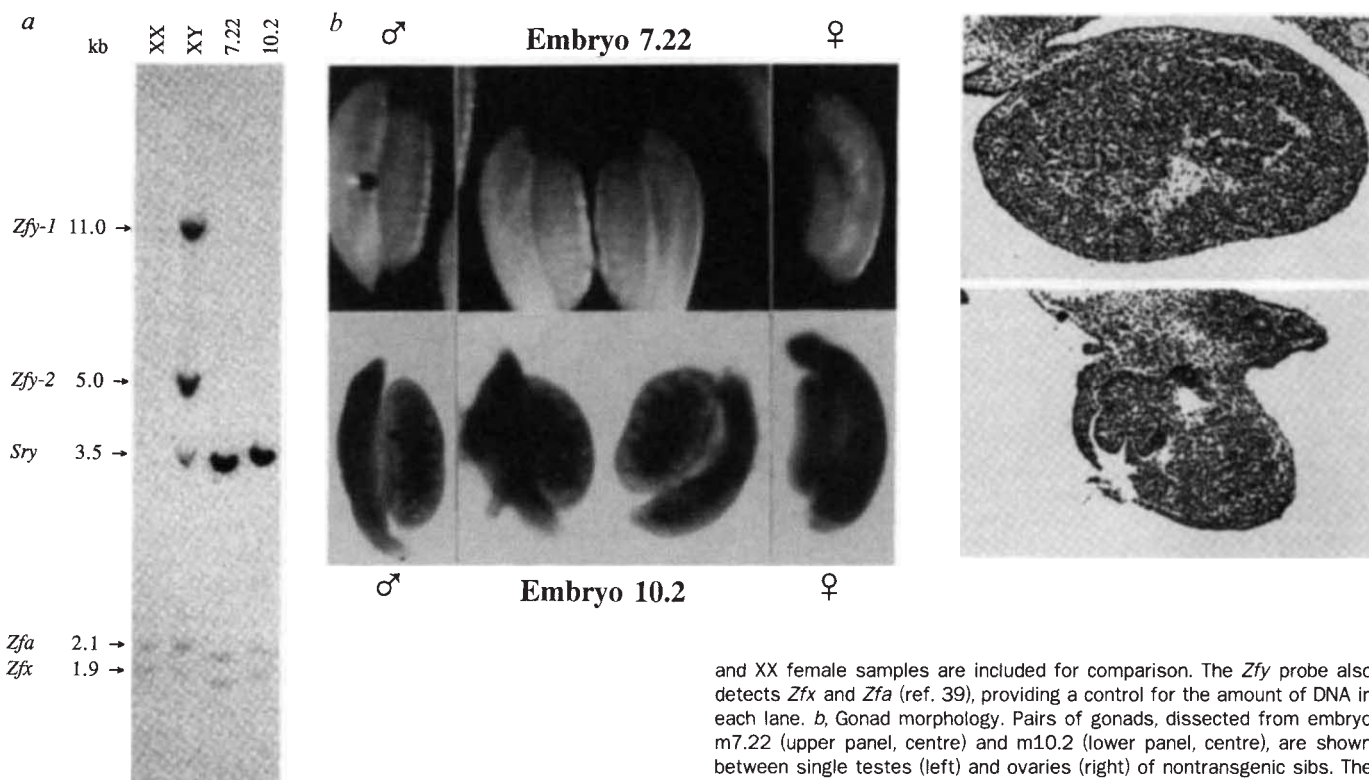


FIG. 2 Analysis of sex-reversed transgenic embryos. a, Southern-blot analysis of DNA from two phenotypic male embryos m7.22 and m10.2. Lack of hybridization to a probe recognizing *Zfy-1* and *Zfy-2* shows the absence of a Y chromosome, whereas the intensity of hybridization to the *Sry* probe demonstrates that they carry multiple copies of the transgene. XY male

and XX female samples are included for comparison. The *Zfy* probe also detects *Zfx* and *Zfa* (ref. 39), providing a control for the amount of DNA in each lane. b, Gonad morphology. Pairs of gonads, dissected from embryo m7.22 (upper panel, centre) and m10.2 (lower panel, centre), are shown between single testes (left) and ovaries (right) of nontransgenic sibs. The gonads of the transgenic embryos show the characteristic stripes associated with testis-cord formation. c, Histology of m7.22 (upper panel) and m10.2 (lower panel) testis sections. The apparent difference in size is due to plane of section. Cord morphology was similar to that of littermates (not shown). METHODS. Southern-blot analysis and probes are described in Table 1 legend. Gonads were photographed whole in PBS, then fixed in 4% paraformaldehyde, dehydrated in ethanol and embedded in paraffin. Sections (7 μ m) were stained in haematoxylin and eosin.

both of these males lacked *Zfy* sequences and were transgenic, with many copies of *Sry* (Fig. 2a). Histological examination showed that their testis-cord formation was normal and that their gonads were indistinguishable from testes of normal XY sib embryos (Fig. 2b, c).

From these experiments we conclude that a 14-kb genomic fragment carrying *Sry* sequences is sufficient to initiate testis development in mice.

To determine the frequency with which f741 gives sex reversal, all the embryos scored as females were examined for the presence of *Sry* sequences by PCR. Two were unequivocally identified as transgenic for *Sry*, and four more were probably mosaics possessing the transgene in a low proportion of cells, as only weak signals for the presence of *Sry* sequences were detected (Table 1). We attribute the finding that not all XX transgenics show sex reversal to such mosaicism or to position effects on *Sry* expression (see below).

Normal adult male phenotype

To test the adult phenotype of *Sry* transgenic mice some of the embryos injected with f741 were allowed to develop to term. A total of 93 animals were born (49 males and 44 females). Southern blotting showed that five of these were transgenic. Two were XY males that did not transmit the transgene and so were uninformative with respect to sex reversal.

One of the transgenics, m33.13, had no Y chromosome as determined by PCR analysis (Fig. 3a) but was externally male (Fig. 3b). He was similar in size and weight to his normal XY male littermates. At about six weeks *post partum*, m33.13 was

caged with females (a maximum of two per night). His copulatory behaviour was normal, mating four times in six days.

The presence of two X chromosomes in a male mouse always results in sterility, as germ cells are prevented from progressing beyond prospermatogonia. This phenomenon has been documented in XX Sxr and XX Sxr' mice which are male owing to the presence of Y-derived sequences including *Tdy* on one of their X chromosomes²²⁻²⁴. It was therefore not surprising that the sex-reversed transgenic mouse m33.13 was also sterile. None of the four females with which he mated became pregnant. In three cases the vaginal plugs were examined for the presence of sperm, but none was found (data not shown). The only difference between m33.13 and a normal XY sibling was in the size of the testes: m33.13 had a testis weight of 17 mg (in the range expected for an XX Sxr' male), as opposed to 76 mg for an XY control littermate. Histological examination of sections of the testes revealed the presence of tubules, with clearly defined and apparently normal populations of Leydig cells, peritubular myoid cells and Sertoli cells, but no cells undergoing spermatogenesis (Fig. 3c).

Internal examination of m33.13 revealed a normal male reproductive tract with no signs of hermaphroditism. This indicates that Sertoli and Leydig cells must have functioned normally in producing anti-Müllerian hormone (AMH) and testosterone, respectively. AMH is required for the elimination of the female Müllerian duct system (oviducts, uterus and upper vagina) and testosterone for the development of the Wolffian duct derivatives (vas deferens and accessory glands such as the seminal vesicles) and male secondary sexual characteristics^{15,25}.

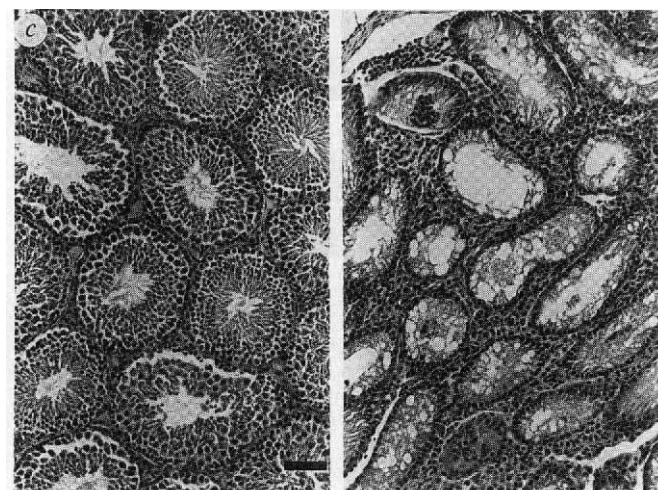
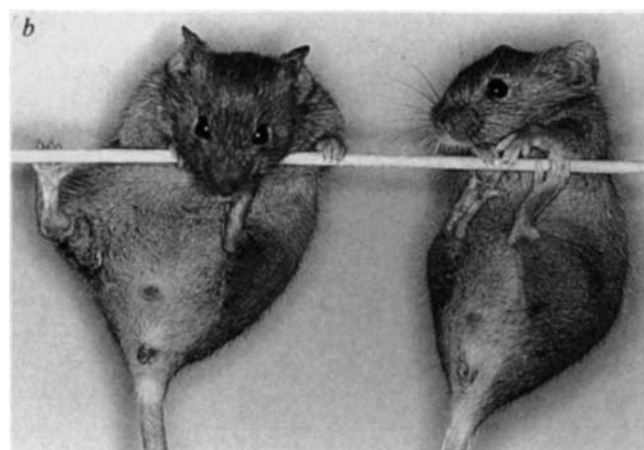
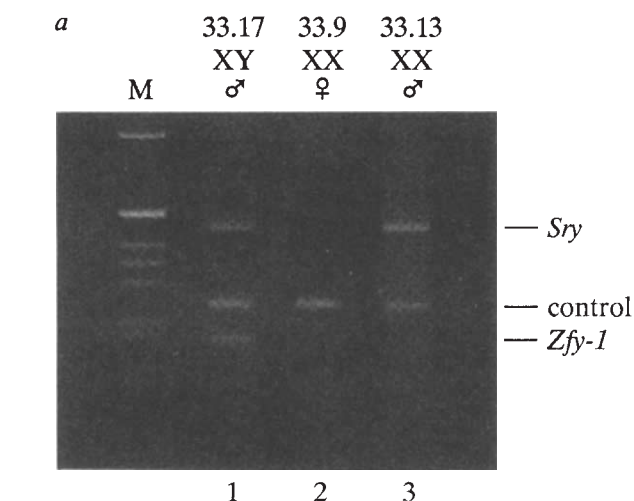


FIG. 3 Analysis of adult sex-reversed transgenic mouse m33.13. *a*, PCR analysis of genomic DNA from m33.13 (lane 3), showing *Sry* and control (myogenin) bands. No band corresponding to *Zfy-1* was seen, demonstrating the lack of a Y chromosome; this result was confirmed by Southern blotting using Y-chromosome probes Y353B (ref. 40) and Sx1 (ref. 41) (not shown). Normal XX female and XY male littermates (33.9, lane 2 and 33.17, lane 1) are shown for comparison. M, marker bands (1,018, 510, 396, 344, 298, 220, 201, 154 and 134 base pairs). *b*, External genitalia of mice 33.17 (left) and 33.13 (right). *c*, Histology of testis sections from mice 33.17 (left) and 33.13 (right). Bar, 90 μ m.

METHODS. For PCR analysis, 0.1 μ g genomic DNA was added to a 50- μ l reaction mixture containing 1.5 mM each dNTP, 50 mM Tris-HCl, pH 9, 15 mM ammonium sulphate, 7 mM MgCl₂, 0.05% Nonidet P-40, 0.5 U *Taq* polymerase (Anglian Biotec) and 500 ng each oligonucleotide primer. Amplification consisted of 30 cycles of 94 °C for 5 s, 65 °C for 30 s and 72 °C for 30 s in a Techne PHC-2 thermocycler. An 8- μ l aliquot was electrophoresed on a 2% agarose-TBE gel. Primers for *Sry* were (5'-3') TCATGAGACTGCCAACCACAG and CATGACCACCACCACCAA (indicated as triangles in Fig. 1) and for *Zfy-1*, CCTATTGCATGGACTGCAGCTTATG and GACTAGACATGTCTTAACATCTGTCC; myogenin primers corresponded to nucleotides 656-675 and 882-901 of the rat complementary DNA sequence⁴². PCR products were 441, 180 and 245 bp, respectively. Testes were processed for histological examination as described in Fig. 2 legend.

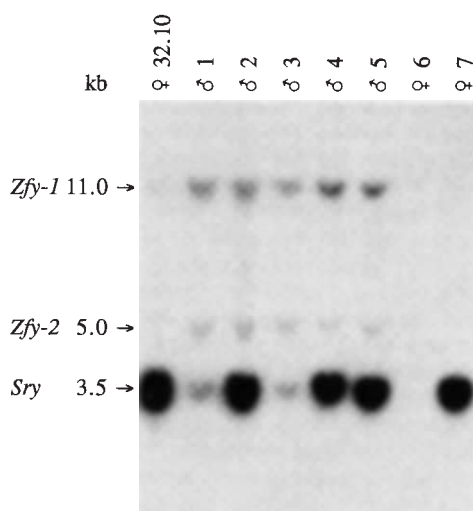


FIG. 4 Southern-blot analysis of offspring derived from transgenic female m32.10. As described in Fig. 2a, lack of hybridization to a probe recognizing *Zfy-1* and *Zfy-2* indicates the absence of a Y chromosome. Founder female m32.10 hybridizes intensely to the probe recognizing *Sry* demonstrating the presence of multiple copies of the transgene. The same band pattern is shown by offspring number 7. Examination of the external genitalia of this animal revealed a normal female phenotype. DNA from the male offspring 1–5 hybridizes to both *Zfy* and *Sry* probes. Three of these have multiple copies of *Sry* and are therefore transgenic.

METHODS. Mouse m32.10 was mated with an F₁ (CBA × C57BL/10) male and biopsies of the resulting offspring's tails made at 3 weeks. Genomic DNA preparation and Southern-blot analysis were as described in Table 1 legend.

A further two XX transgenics, m32.10 and m33.2, showed an external female phenotype, yet both carried many copies of *Sry*. These mice have produced offspring and so have functional reproductive tracts and ovaries. They also provide further evidence, along with the transgenic XX female fetuses, that f 741 does not always cause sex reversal. Although there could be subtle rearrangements of the *Sry* gene making it non-functional, the possibility of this occurring in all these cases is remote. There are two more probable explanations. First, these females could be mosaic for the transgene, with only a small proportion of the cells making up the somatic portion of the genital ridge carrying functional *Sry* gene copies. Analysis of XX ↔ XY chimaeras suggests that females or hermaphrodites develop if less than about 30% of cells are XY^{17,26,27}. Secondly, the expression of the transgene could be affected by the position at which it integrates. Except for a few cases where locus-controlling regions are present, expression of transgenes almost always depends on their chromosomal location²⁸. These two alternatives can be examined by breeding from the adult XX transgenic females. Mouse m33.2 has not yet produced transgenic offspring. However, m32.10 has transmitted the transgene to female offspring (Fig. 4), suggesting that it is not mosaic.

Human *SRY* does not function in mice

Mouse *Sry* and human *SRY* have a highly similar nucleotide

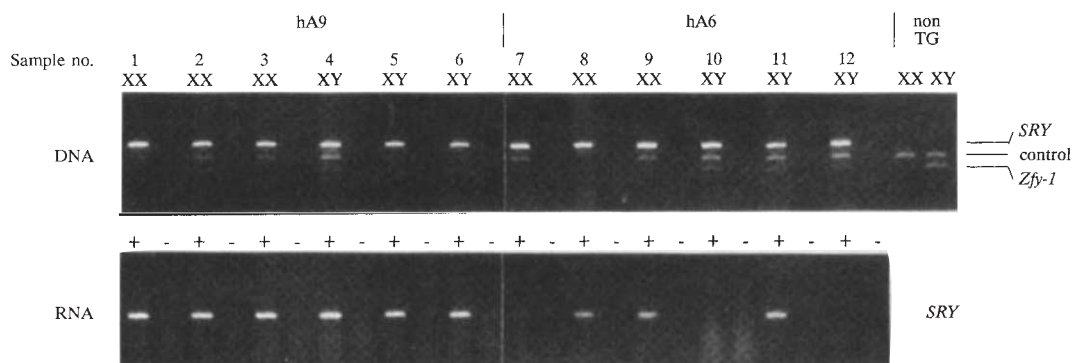
sequence in the region encoding the putative DNA-binding domain⁹. However, the two domains differ in 23 of their 79 amino-acid residues, with only two of the differences being conservative. The specificity of interaction of *SRY/Sry* with other genes in the sex determination pathway presumably depends on the structure of this domain. That only a single amino-acid substitution can lead to failure of testicular development in an XY individual¹³ suggests that even a subtle alteration in the *SRY* protein could disrupt its action. In addition, the nucleotide sequences of the mouse and human genes diverge strikingly outside the region encoding the putative DNA-binding domain⁹. We therefore tested whether the human *SRY* gene is as effective as its murine counterpart in causing sex reversal in mice.

The DNA used for pronuclear injection was a 25-kb *Bam*HI–*Sal*I fragment representing human Y chromosomal DNA around *SRY*, isolated from a cosmid clone cAMF (ref. 29). This fragment includes roughly 12.5 kb of Y-unique sequence upstream, and 5 kb downstream, of the *SRY* conserved domain. The remaining 6 kb represents sequences from the pseudoautosomal region, which is common to the X and Y chromosomes (Fig. 1).

The ability of human *SRY* to cause sex reversal was assessed in animals representing three independent integrations of the transgene. Two of these were lines derived from XY founder

FIG. 5 Expression of human *SRY* in transgenic mouse embryos. Three male and three female embryos from each of two lines (hA9 and hA6) transgenic for *SRY* were analysed at 11.5 d.p.c. PCR analysis of genomic DNA (upper panel) showed that all embryos (lanes 1–12) were transgenic for *SRY*. Samples 4–6 and 10–12 showed bands corresponding to *Zfy-1*, confirming that they were XY, unlike samples 1–3 and 7–9 which were XX. Non-transgenic controls (non TG) are shown on the right. Oligonucleotide primers for myogenin were included in each analysis as a control. Below each lane is shown a PCR analysis of reverse-transcribed RNA extracted from the urogenital ridges of the same embryos (lower panel). Reverse transcription was performed on each RNA sample with (+) and without (–) reverse transcriptase, to demonstrate that the observed bands were due to the presence of *SRY* transcripts and not contaminating DNA. Bands corresponding to *SRY* expression were seen in samples 1–9 and 11.

METHODS. RNA was extracted from pairs of urogenital ridges and reverse-



transcribed in small-scale reactions as described²¹, using half the yield from each pair of ridges in the + and – reverse-transcriptase reactions. Genomic DNA or reverse-transcription products were added to PCR reactions and amplified as described in Fig. 3 legend. For DNA analysis, *SRY* primers used were (5'–3') GATCAGCAAGCAGCTGGGATACAGTG and CTGTAGCGGTCCCGT-TGCTGCGGTG (336-bp product, solid triangles in Fig. 1); for RNA analysis, CAGGAGGCACAGAAATTACAGGCTGC and ACAGTCATCCCTGTACAACCT-GTTGTC (174-bp product; open triangles in Fig. 1) were used.

transgenics, hA6 and hA9. These founders transmitted *SRY* to about half of their offspring: 8 out of 17 hA6 and 13 out of 36 hA9 embryos assayed were transgenic. We analysed progeny of these lines at 14.5 d.p.c., and found no evidence of testis-cord formation in XX transgenic fetuses, demonstrating that neither of the integrations in these lines could cause sex reversal. A third integration was represented by a single XX transgenic founder embryo; this too was phenotypically female.

Having transgenic lines that transmit human *SRY* to their offspring allowed us to examine expression of the transgene in developing gonads, and in the only other known site of *Sry* expression, the adult testis^{8,12}. Genital ridges were dissected from hA6 and hA9 fetuses at 11.5–12 d.p.c., when mouse *Sry* is expressed¹². PCR analysis of RNA extracted from these samples shows that *SRY* transcripts were present in transgenic XX fetuses that were not sex-reversed (Fig. 5). Curiously, not all hA6 XY fetuses expressed the transgene (Fig. 5); this could be due to variation in developmental stage. The level of *SRY* expression in the genital ridges was estimated to be several times that of the endogenous *Sry* gene, and was greater than that seen in transgenic XY adult testis material (not shown).

Clearly a lack of transcription in the genital ridge cannot account for the failure of *SRY* to give sex reversal in mice. It is formally possible that the *SRY* mRNA is not correctly processed or translated. Alternatively, the protein product could be unstable in mouse cells. However, it is more likely that differences in sequence result in the human *SRY* protein failing to interact with other regulatory proteins or target genes in mouse cells. This hypothesis could be tested by exchanging the human and mouse open reading frames.

Discussion

The experiments described here demonstrate that a 14-kb mouse genomic DNA fragment containing *Sry* is sufficient to direct the formation of testes in XX transgenic embryos and subsequently to give rise to full phenotypic sex reversal in an XX transgenic adult. Complete sequencing of f741, as well as cross-hybridization experiments to human DNA, have failed to detect any gene sequences other than *Sry* (D. Jackson and A. Sinclair, unpublished results). Although previous data provided compelling evidence to implicate *Sry* in the process of testis determination, this study suggests that *Sry* is the only Y-linked gene required to give rise to male development, and we propose that *Sry* is *Tdy*.

The ability of a 14-kb fragment containing *Sry* to cause sex reversal suggests that this fragment contains the entire *Sry* gene, including all the regulatory elements required for appropriate

embryonic expression. The transgenic system can be used to localize further these regulatory elements within the 14-kb fragment by microinjecting progressively smaller constructs. The only other site of *Sry* expression is in adult testis, probably in the germ-cell component¹². It will be interesting to see if the 14-kb fragment f741 also contains the regulatory information required for the switch from expression in the somatic part of the embryonic gonad to expression in adult testis associated with germ cells. Our finding that the human *SRY* gene is expressed at both stages in transgenic mice is consistent with the appropriate regulatory sequences being present within the 25 kb of DNA containing the gene. It also indicates that the mechanisms regulating *Sry*/*SRY* expression have been conserved between humans and mice.

Although we have shown that *Sry* alone can promote testicular development in the absence of other Y-linked genes, sex reversal does not always occur. The most likely explanation for this is that the *Sry* transgene is sensitive to position effects. This variability may mirror the situation in human XX individuals carrying small portions of the Y chromosome including *SRY*. Palmer *et al.*³⁰ described four such cases, each of whom had inherited about 35 kb of Y-unique sequences on their paternal X chromosome, two of whom were only partially sex-reversed. Jäger *et al.*³¹ also describe an XX hermaphrodite with a similar small portion of the Y. In these cases *SRY* may be affected not only by adjacent DNA sequences in its new chromosomal location, but also by the spread of X-inactivation. There are several precedents for the latter in the mouse; for example females or hermaphrodites frequently develop instead of males when the *Sxr* fragment is present only on the inactive X chromosome³². There is of course no reason to believe that X-inactivation is involved in expression of the transgene considered here. Nevertheless, when additional transgenic mice are analysed, we would expect to find instances of partial sex reversal due to position effects, where the level of *Sry* expression is close to a critical threshold. It will be important to understand what this threshold means in the process of testis determination.

Sry acts over a short time to initiate testis development. It must do this through interaction with other genes, some of which will be involved in the regulation of *Sry*, others of which will be downstream targets of *Sry*. These other genes must map elsewhere in the genome, because *Sry* is shown here to be the only Y-linked gene required to bring about male development in mice. Mutations in some of these genes could explain cases of male development in XX individuals lacking *SRY*³⁰, and XY females where *SRY* is intact^{13,14,33,34}. Using molecular genetic techniques to work stepwise from *Sry* it should now be possible to identify these other genes. □

Received 28 March; accepted 10 April 1991.

- McLaren, A. *Trends Genet.* **4**, 153–157 (1988).
- McLaren, A. *Phil. Trans. R. Soc.* **322**, 3–9 (1988).
- Jacobs, P. A. & Strong, J. A. *Nature* **183**, 302–303 (1959).
- Ford, C. E. *et al. Lancet* **i**, 711–713 (1959).
- Welshons, W. J. & Russell, L. B. *Proc. natn. Acad. Sci. U.S.A.* **45**, 560–566 (1959).
- Jost, A. *Archs. Anat. microsc. Morph. exp.* **36**, 271–315 (1947).
- Jost, A. *et al. Recent Prog. Horm. Res.* **29**, 1–41 (1973).
- Sinclair, A. H. *et al. Nature* **346**, 240–244 (1990).
- Gubbay, J. *et al. Nature* **346**, 245–250 (1990).
- Lovell-Badge, R. H. & Robertson, E. *Development* **109**, 635–646 (1990).
- Gubbay, J. *et al. Development* **109**, 647–653 (1990).
- Koopman, P. *et al. Nature* **348**, 450–452 (1990).
- Berta, P. *et al. Nature* **348**, 448–450 (1990).
- Jäger, R. *et al. Nature* **348**, 452–454 (1990).
- Jost, A. & Magre, S. *Phil. Trans. R. Soc.* **322**, 55–61 (1988).
- Eicher, E. M. *Phil. Trans. R. Soc.* **322**, 109–118 (1988).
- Eicher, E. M. *et al. Cytogenet. Cell Genet.* **28**, 104–115 (1980).
- Burgoyne, P. S., Tam, P. L. & Evans, E. P. *J. Reprod. Fert.* **68**, 387–393 (1983).
- Mardon, G. *et al. Science* **243**, 78–80 (1989).
- Nagamine, C. M. *et al. Science* **243**, 80–83 (1989).
- Koopman, P. *et al. Nature* **342**, 940–942 (1989).
- Cattanach, B. M., Pollard, C. E. & Hawkes, S. G. *Cytogenetics* **10**, 318–337 (1971).
- Burgoyne, P. S., Levy, E. R. & McLaren, A. *Nature* **320**, 170–172 (1986).
- Sutcliffe, M. J. & Burgoyne, P. S. *Development* **107**, 373–380 (1989).
- Josso, N. in *Mechanisms of Sex Differentiation in Animals and Man* (eds Austin, C. R. & Edwards, R. G.) 165–203 (Academic, London, 1981).

- Burgoyne, P. S. *et al. Development* **102**, 443–450 (1988).
- Palmer, S. J. & Burgoyne, P. S. *Development* **111**, 1017–1020 (1991).
- Grosfeld, F. *et al. Cell* **51**, 975–985 (1987).
- Ellis, N. A. *et al. Nature* **337**, 81–84 (1989).
- Palmer, M. S. *et al. Nature* **342**, 937–939 (1989).
- Jäger, R. J. *et al. Hum. Genet.* **85**, 666–668 (1990).
- McLaren, A. & Mork, M. *Nature* **300**, 446–448 (1982).
- Scherer, G. *et al. Hum. Genet.* **81**, 291–294 (1989).
- Fredga, K. *Phil. Trans. R. Soc.* **322**, 83–95 (1988).
- Russell, L. B., in *Chemical Mutagens. Principles and Methods for their Detection* Vol. 4 (ed. Hollaender, A.) 55–91 (Plenum, New York, London, 1976).
- Lovell-Badge, R. H., in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach* (ed. Robertson, E. J.) 153–182 (IRL, Oxford, 1987).
- Maniatis, T., Fritsch, E. F. & Sambrook, J., eds., *Molecular Cloning* (Cold Spring Harbor Laboratory, New York, 1982).
- Hogan, B., Costantini, F. & Lacy, E., eds., *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory, New York, 1986).
- Ashworth, A. *et al. EMBO J.* **9**, 1529–1534 (1990).
- Bishop, C. E. *et al. Nature* **315**, 70–72 (1985).
- Roberts, C. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 6646–6649 (1988).
- Wright, W. E., Sassoon, D. A. & Lin, V. K. *Cell* **56**, 607–617 (1989).

ACKNOWLEDGEMENTS. We thank our colleagues for encouragement and helpful criticism, and in particular D. Jackson and A. Sinclair for access to unpublished results. The help of the photography and histology services at NIMR is gratefully acknowledged.

Ha-Ras augments c-Jun activity and stimulates phosphorylation of its activation domain

Bernard Binétruy^{*†}, Tod Smeal^{*‡} & Michael Karin^{*§}

^{*} Departments of Pharmacology and [‡] Biology, Center for Molecular Genetics, School of Medicine, 0636, University of California San Diego, La Jolla, California 92093, USA

[†] Present address: Institut Curie-Biologie, Centre Universitaire, 91405 Orsay, France

[§] To whom correspondence should be addressed

Ha-Ras augments c-Jun-mediated transactivation by potentiating the activity of the c-Jun activation domain. Ha-Ras also causes a corresponding increase in phosphorylation of specific sites in that part of the c-Jun protein. A Ha-Ras-induced protein kinase cascade resulting in hyperphosphorylation of the c-Jun activation domain could explain how these oncoproteins cooperate to transform rat embryo fibroblasts.

and transient expression of transforming oncogenes such as activated Ha-ras^{20,21}. Stimulation of AP-1 activity by transforming oncogenes is not fully understood but is probably important in the neoplastic process because AP-1 is the major transcription factor whose activity is augmented by cellular transforming oncogenes²², possibly accounting for altered patterns of gene expression in malignant cells. It was suggested that the effect of Ha-Ras on AP-1 activity was through induction of *c-fos* and *c-jun*^{22,23}. But induction of *c-fos* by Ha-Ras is transient²⁴ and there is no evidence for elevated *c-fos* expression in most transformed cells. The findings that Ha-ras cooperates not only with *c-jun*³ but also with TPA²⁵, an inducer of *c-jun*, and that cells resistant to transformation by *v-fos* are also resistant to Ha-ras²⁶ and *c-jun*²⁷, led us to examine the effect of activated Ha-Ras on c-Jun activity. We found that expression of activated Ha-Ras caused a marked increase in transcriptional activation by c-Jun in various cell types, including REF, independently of *c-fos* induction. This stimulation results from the increased potency of the c-Jun N-terminal activation domain and correlates with its increased phosphorylation.

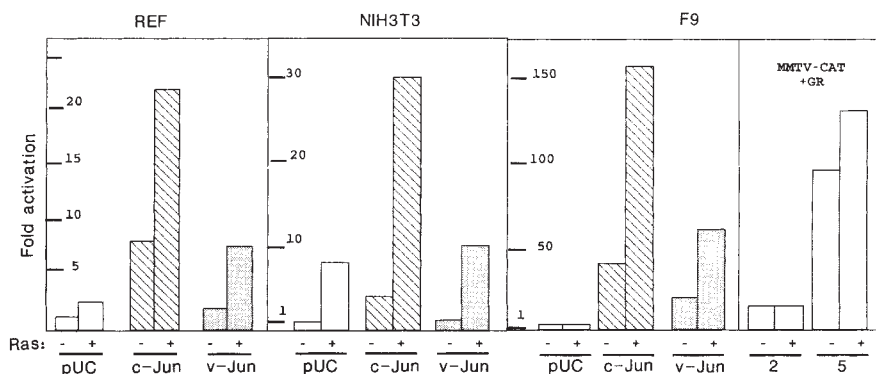
Ha-Ras stimulates transactivation by c-Jun

Stimulation of c-Jun activity by Ha-Ras could account for the oncogenic cooperation between *c-jun* or TPA and Ha-Ras^{3,25}. A CAT (chloramphenicol acetyltransferase) reporter gene controlled by a TRE-containing collagenase promoter (−73Col-CAT)⁵ was used to examine transactivation by c-Jun in REF cells. Ha-Ras protein was provided by pZIPneoRas which encodes an activated form (leu61) of human Ha-Ras²⁸. Alone, pZIPneo-Ras stimulated CAT activity two- to threefold (Fig. 1). Although of smaller magnitude, this effect is consistent with stimulation of AP-1 activity of Ha-Ras in established cell lines^{20,21}. Ha-Ras also increased transactivation by c-Jun three- to fourfold. Transactivation of v-Jun was also stimulated but to

PROTO-ONCOGENE *c-jun* encodes a nuclear protein capable of transforming either chicken cells^{1,2}, immortalized rat fibroblasts³ or primary rat embryo fibroblasts (REF) in cooperation with activated Ha-Ras (ref. 3). The c-Jun protein is a major component of the transcription factor AP-1 (refs 4, 5), originally shown to mediate phorbol ester tumour promoter (12-O-tetradecanoylphorbol-13-acetate; TPA)-induced expression of responsive genes through the TPA-response element (TRE)^{6,7}, a specific site in their promoters. Other AP-1 components belong either to the Jun (JunB, JunD) or Fos (c-Fos, FosB, Fra1) families. The Jun proteins form homo- and heterodimers which bind the TRE, but the Fos proteins are active only as heterodimers with any of the Jun proteins^{1,8}. Among the Jun proteins, only c-Jun activates transcription of target genes containing a single TRE in the absence of Fos^{9–11}.

The *jun* and *fos* genes are induced by external stimuli such as TPA and growth factors^{12–16}. Induction of *c-jun* by TPA is thought to result from a positive autoregulatory mechanism¹⁶, and to involve dephosphorylation of preexisting c-Jun at inhibitory phosphorylation sites next to its DNA-binding domain¹⁷. AP-1 activity is also induced by neoplastic transformation^{18,19}

FIG. 1 Ha-Ras augments transactivation by c-Jun. REF (0.5 µg per plate), NIH3T3 (0.5 µg per plate) or F9 (2 µg per plate) cells were transfected with the AP-1-dependent −73Col-CAT reporter in the presence or absence of pZIPneoRas (5 µg per plate) and RSV-c-Jun (0.5 µg per plate in REF and NIH3T3, 2 µg per plate in F9) or RSV-v-Jun (2 µg per plate in all cell types) expression vectors, as indicated. The cells were collected 24–48 h after transfection and the CAT activity determined. The values shown represent averages from three experiments in each of the cell types. As a control for specificity of the Ha-Ras effect, the glucocorticoid receptor (GR)-dependent reporter MMTV-CAT (1 µg per plate) was transfected into F9 cells in the presence of either 2 or 5 µg per plate of a GR expression vector³⁴ and 10^{−6} M dexamethasone in the presence or absence of pZIPneoRas (5 µg per plate). CAT activity was determined 24 h after transfection. The results are from one experiment done in duplicate. METHODS. All the expression vectors and reporters used in these experi-



ments have been described previously^{5,6,34}. Primary REF cultures were prepared from day-14 Fisher rat embryos by established procedures³ and were used between 1 and 3 passages. NIH3T3 and F9 cells were cultured as described^{9,11}. Transfections were done as described^{5,9}.

a lesser extent. The effect of Ha-Ras was mediated through the AP-1 binding site: a -63Col-CAT reporter lacking the TRE (ref. 5) did not respond to either Ha-Ras alone or Ha-Ras with c-Jun (data not shown). Ha-Ras did not increase c-Jun expression because an RSV-CAT reporter controlled by the same promoter used to express c-Jun was not induced (data not shown) and, more directly, the rate of c-Jun synthesis was unchanged (Fig. 3a).

pZIPneo-Ras also augmented transactivation by c-Jun four- to sevenfold in NIH3T3 and F9 cells (Fig. 1). As reported^{20,21}, Ha-Ras alone led to a marked increase in AP-1 activity in NIH3T3 cells, but not in F9 cells. As F9 cells do not express c-Jun^{9,11}, it would seem that stimulation of AP-1 activity by Ha-Ras requires c-Jun expression. As shown earlier²⁹, v-Jun did not stimulate AP-1 activity in NIH3T3 cells, even in the presence of Ha-Ras, but in F9 cells v-Jun activated -73Col-CAT and was responsive to Ha-Ras. The basis for the different activities of c-Jun and v-Jun in these cells is not yet clear. Although v-Jun was reported to be a more potent activator than c-Jun, these results were specific for HeLa cells³⁰ and in three other mammalian cell lines (F9, NIH3T3, HepG2) and, most importantly, primary REF cultures, c-Jun is more potent than v-Jun²⁹⁻³¹.

We also tested the effect of Ha-Ras on another activator, the glucocorticoid receptor. In F9 cells, Ha-Ras had a marginal effect on glucocorticoid receptor activity (Fig. 1), but in NIH3T3 and REF cells Ha-Ras inhibited activity (data not shown). This well documented inhibition³² is likely to be mediated by induction of c-Jun and c-Fos³³⁻³⁵. Ha-Ras also had a marginal effect on GAL4/VP16 (data not shown) or GHF-1 (see Fig. 5) activity. Thus, as previously shown^{18,20}, Ha-Ras is not a general stimulator of transcription.

Stimulation of c-Jun activity is Fos-independent

As transient expression of Ha-Ras induces *c-fos*²⁴, the stimulation of either endogenous AP-1 or transfected c-Jun by Ha-Ras could be explained by heterodimerization between c-Fos and c-Jun³⁶. To assess the contribution of c-Fos to stimulation of c-Jun activity we first examined whether Ha-Ras affects the activity of JunB or JunD, both of which are highly dependent on c-Fos for transactivation^{10,11}. Neither JunB (Fig. 2a) nor JunD (data not shown) were affected by Ha-Ras, although their activity was markedly enhanced by cotransfected c-Fos expression vector. We also examined two dimerization mutants of c-Jun. M15, in which two leucines in the c-Jun leucine zipper are replaced by phenylalanines, cannot form homodimers but can form heterodimers with c-Fos³⁷. M3, in which Lys 288 thought to be involved in salt-bridge formation with c-Fos is replaced by a glutamate, is more active as a homodimer and is insensitive to further stimulation by c-Fos³⁷. We find that

although M15 is augmented by c-Fos, it is not responsive to Ha-Ras (Fig. 2b). M3, on the other hand, was insensitive to c-Fos but fully responsive to Ha-Ras. Therefore, the stimulation of c-Jun activity by Ha-Ras seen in these experiments is not mediated by c-Fos.

Ha-Ras stimulates phosphorylation of c-Jun

Ha-Ras is known to induce *c-jun*²³ and it was therefore possible that the augmentation of c-Jun activity resulted from induction of endogenous c-Jun protein which then acted with exogenous c-Jun. But Ha-Ras had no effect on the level of c-Jun expression in transfected REF (Fig. 3a) or F9 cells (data not shown). We therefore considered that Ha-Ras may stimulate c-Jun activity post-translationally. Cotransfection with Ha-Ras led to a considerable and specific increase in the level of c-Jun phosphorylation (Fig. 3a). As the phosphorylation of proteins trapped in the immunoprecipitates was not affected, the Ha-Ras effect on c-Jun phosphorylation is not due to increased uptake of ³²P-orthophosphate by the cells.

AP-1 activity is also elevated in Ha-Ras-transformed fibroblasts²⁰. In the same cell lines used by Wasylyk *et al.*²⁰, Ha-Ras transformation leads to a specific 4.5-fold increase in c-Jun synthesis and a 35-fold increase in c-Jun phosphorylation (Fig. 3b). The stimulation of c-Jun phosphorylation is much higher than the general increase in protein phosphorylation in Ha-Ras transformed cells, which amounts to 2-fold for two of the background proteins present in the c-Jun immunoprecipitates. After normalizing for this general increase and its elevated rate of synthesis, we find that c-Jun phosphorylation is 4-fold higher after transformation by Ha-Ras.

Phosphopeptide maps (Fig. 4) indicate that the effect of Ha-Ras expression on c-Jun phosphorylation is site-specific. We find that Ha-Ras increases phosphorylation of sites x and y, located in the N-terminal half of c-Jun¹⁷, at least 4-fold. Another N-terminal site, z, is not significantly affected. As reported¹⁷, these sites contain only phosphoserines (data not shown). The effect of Ha-Ras on the three C-terminal sites, which are phosphorylated *in vitro* by GSK3 (ref. 17), is more complex. These sites reside within a single tryptic peptide whose triphosphorylated form corresponds to spot a, its diphosphorylated forms account for doublet b, and its monophosphorylated forms migrate as spot c. Ha-Ras led to a 2-fold increase in the mono- and diphosphorylated forms and no change in the triphosphorylated form. Assuming that these sites are phosphorylated by a single protein kinase¹⁷, these changes are consistent with a simultaneous increase in the activity of this protein kinase and the putative phosphatase that acts on these sites. Activity of this phosphatase seems to increase after protein kinase C (PKC) activation and results in site-specific dephosphorylation and

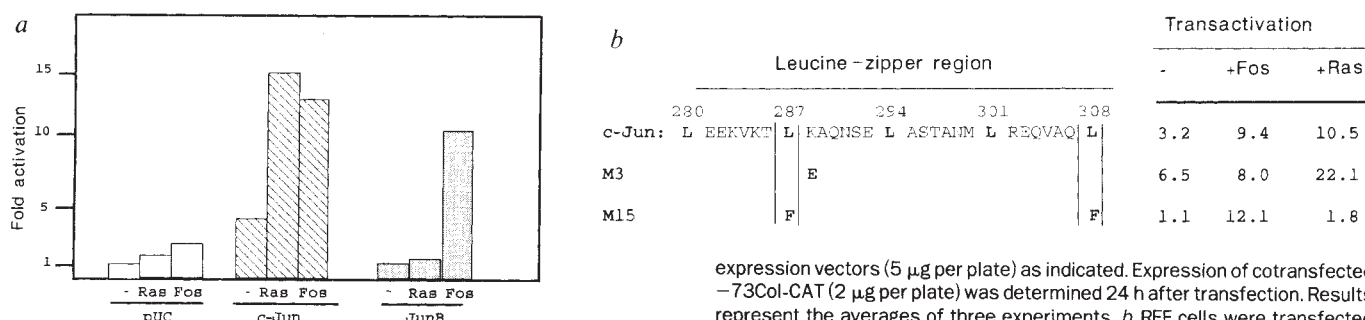


FIG. 2 Stimulation of c-Jun activity by Ha-Ras is independent of Fos. *a*, F9 cells were transfected with RSV-c-Jun (0.5 μ g per plate) and RSV-JunB (5 μ g per plate) expression vectors (these amounts of the c-Jun and JunB vectors were chosen because they led to similar levels of transactivation in the presence of c-Fos) in the presence or absence of Ha-Ras and c-Fos

expression vectors (5 μ g per plate) as indicated. Expression of cotransfected -73Col-CAT (2 μ g per plate) was determined 24 h after transfection. Results represent the averages of three experiments. *b*, REF cells were transfected with wild-type c-Jun and the M3 and M15 leucine-zipper mutants (1.5 μ g per plate) in the presence or absence of c-Fos and Ha-Ras expression vectors (3 μ g per plate) as indicated. Expression of -73Col-CAT was determined 16 h after transfection and the results shown are the averages of three experiments. The sequences of the wild-type and mutant leucine-zippers are shown. Sequences of mutant leucine-zippers shown in single-letter amino-acid code.

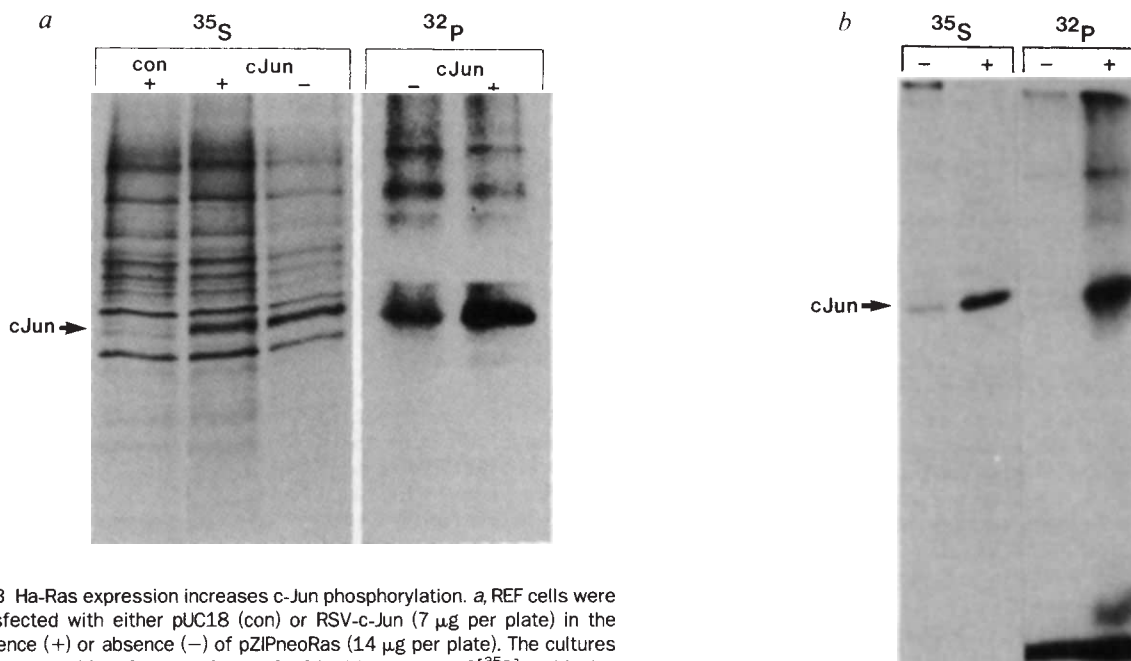


FIG. 3 Ha-Ras expression increases c-Jun phosphorylation. *a*, REF cells were transfected with either pUC18 (con) or RSV-c-Jun (7 µg per plate) in the presence (+) or absence (-) of pZIPneoRas (14 µg per plate). The cultures were labelled 12 h after transfection for 2 h with a mixture of [^{35}S]methionine and [^{35}S]cysteine (Tran[^{35}S]label). Parallel cultures were labelled with [^{32}P]orthophosphate for 5 h. The levels of c-Jun synthesis and phosphorylation were determined by radioimmunoprecipitation with a c-Jun-specific antipeptide antibody. *b*, Subconfluent cultures of FR3T3(-) and Ha-Ras-transformed FR3T3(+) cells were labelled for 2.5 h with either Tran[^{35}S]label or [^{32}P]orthophosphate. The levels of c-Jun synthesis and phosphorylation were determined by radioimmunoprecipitation of lysates derived from an equal number of cells, using a c-Jun-specific antipeptide antibody. The increase in phosphorylation of c-Jun and a protein that contaminates the c-Jun immunoprecipitates is indicated on the right-hand panel. These numbers come from three experiments in which the radioactivity in each band was directly quantitated using an Ambis β counter. Similar results

were also obtained by densitometric analysis of the autoradiograms. **METHODS.** Transfected cell cultures on 100-mm plates were rinsed twice before labelling with serum-free labelling medium and incubated with either 5 mCi ml $^{-1}$ [^{32}P]orthophosphate (ICN) or 150 µCi ml $^{-1}$ Tran[^{35}S]label (from ICN) in 2 ml DMEM lacking either sodium phosphate or L-methionine, respectively. c-Jun proteins were immunoprecipitated from cells lysed in RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 10 mM Tris-HCl pH 8.0 and 0.1 mM PMSF). Lysates were cleared with preimmune serum and c-Jun protein immunoprecipitated with rabbit antiserum directed against a C-terminal peptide of human c-Jun corresponding to AA 316-331 (VNSGCQLMLTQQLQTF) as described 17 .

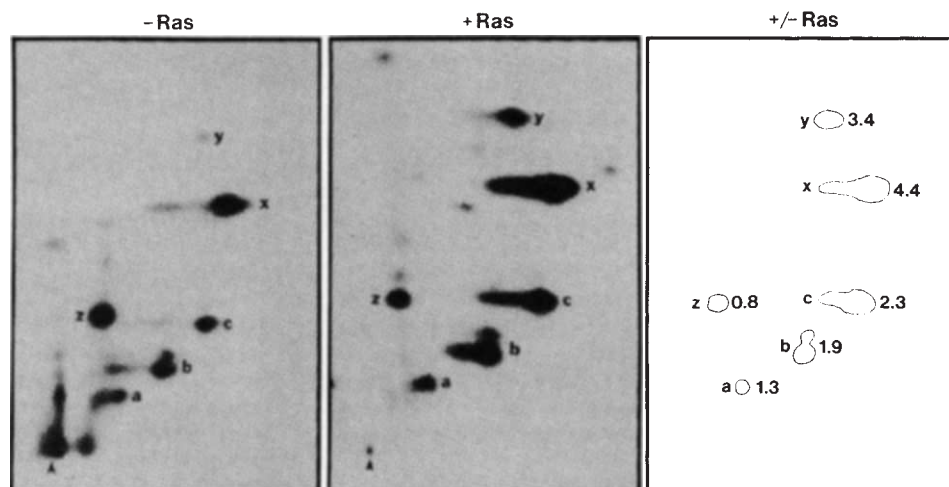
elevated c-Jun DNA binding activity 17 . As the half-life of c-Jun (1.5-2 h; W. Boyle, personal communication) is considerably shorter than the labelling period (5 h), the effect of Ha-Ras on c-Jun phosphorylation is unlikely to be due to differential turnover. In addition, shortening of the labelling period to 2.5 h (Figs 5 and 6) did not affect the observed phosphorylation pattern. Other mapping experiments indicate that c-Jun is also hyperphosphorylated on sites x and y in Ha-Ras-transformed

FR3T3 cells. Ha-Ras transformation also led to a shift of the GSK3 phosphorylation cluster towards its monophosphorylated forms (data not shown).

Effect of Ha-Ras on c-Jun activation domain

These results suggested that it is sites within the N-terminal half of c-Jun, the location of its activation domain 31 , whose phosphorylation is stimulated by Ha-Ras. To determine whether

FIG. 4 Stimulation of c-Jun phosphorylation by Ha-Ras is site-specific. ^{32}P -labelled c-Jun protein was isolated from equal numbers of REF cells transiently transfected with RSV-c-Jun in the presence or absence of pZIPneoRas as indicated in Fig. 3a. After SDS-PAGE the c-Jun protein was eluted and subjected to tryptic digestion. The tryptic digests were applied to thin-layer plates and resolved in the horizontal dimension by electrophoresis at pH 1.9 (anode to the left) and the vertical dimension by ascending chromatography. The phosphopeptides were detected by autoradiography after which they were eluted and counted. The rightmost panel shows the ratio of ^{32}P content in each of the c-Jun phosphopeptides in the presence and absence of Ha-Ras expression.



METHODS. Cell labelling and immunoprecipitation of ^{32}P -labelled c-Jun were as described above. Equal amounts of radioactivity were loaded on an SDS-polyacrylamide gel and after elution from the gel the labelled c-Jun

protein samples were digested to completion with trypsin and subjected to two-dimensional phosphopeptide mapping as previously described 17 .

Ha-Ras affects the c-Jun activation domain we used the chimaeric activator Jun-GHF1, in which the c-Jun activation domain is fused to the DNA binding domain of GHF-1, a pituitary-specific activator^{30,38}. Ha-Ras expression increased activation of the -90GHF1-CAT(Δ AP-1) reporter by Jun-GHF1 5-fold (Fig. 5a). This stimulation cannot be explained by induction of endogenous c-Jun because cotransfection with RSV-c-Jun did not affect transactivation by Jun-GHF1. A reporter that lacks a GHF-1 binding site³⁹ was not activated by Jun-GHF1 even in the presence of Ha-Ras (data not shown).

Under these conditions GHF-1 itself was a weak activator and only marginally responsive to Ha-Ras (Fig. 5a).

Stimulation of Jun-GHF1 activity by Ha-Ras correlates with increased phosphorylation of Jun-GHF1. Ha-Ras did not affect the rate of either Jun-GHF1 or GHF-1 synthesis (Fig. 5b), or the total amount of Jun-GHF1 (Fig. 5c), but it did cause a fivefold increase in Jun-GHF1 phosphorylation (Fig. 5b), although GHF-1 itself was not phosphorylated. The phosphopeptide maps in Fig. 5d confirmed that this phosphorylation occurred on sites derived from c-Jun. The sites phosphorylated

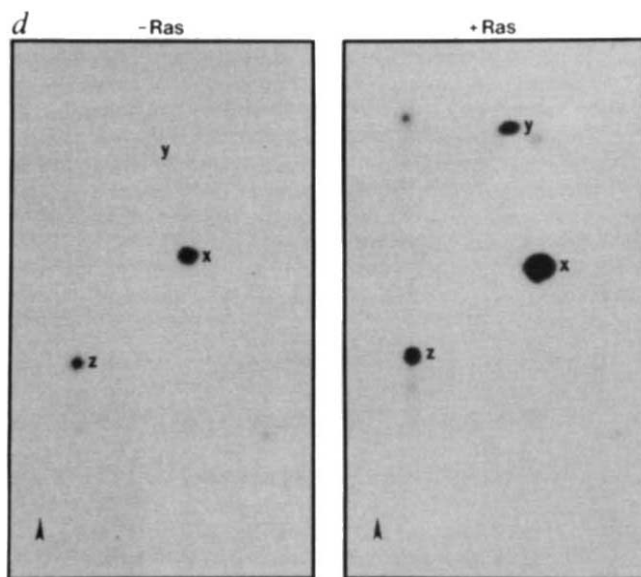
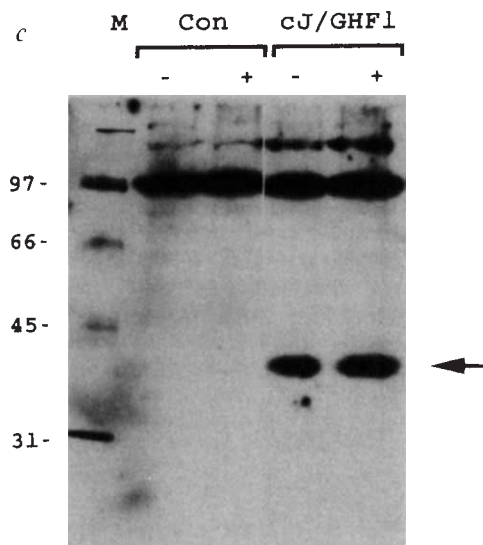
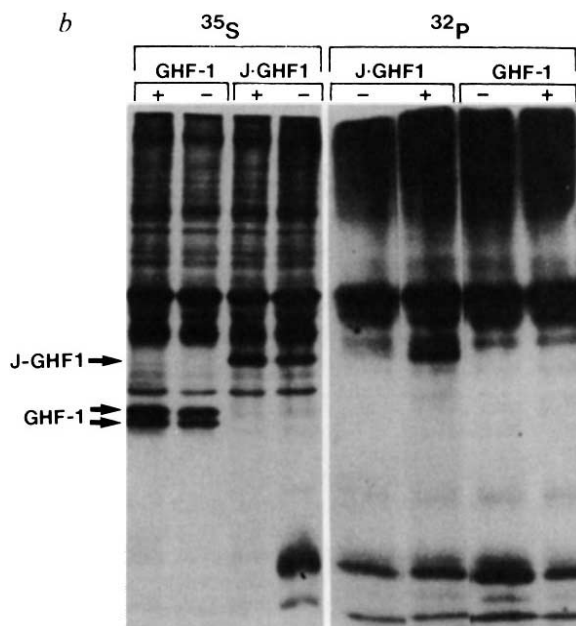
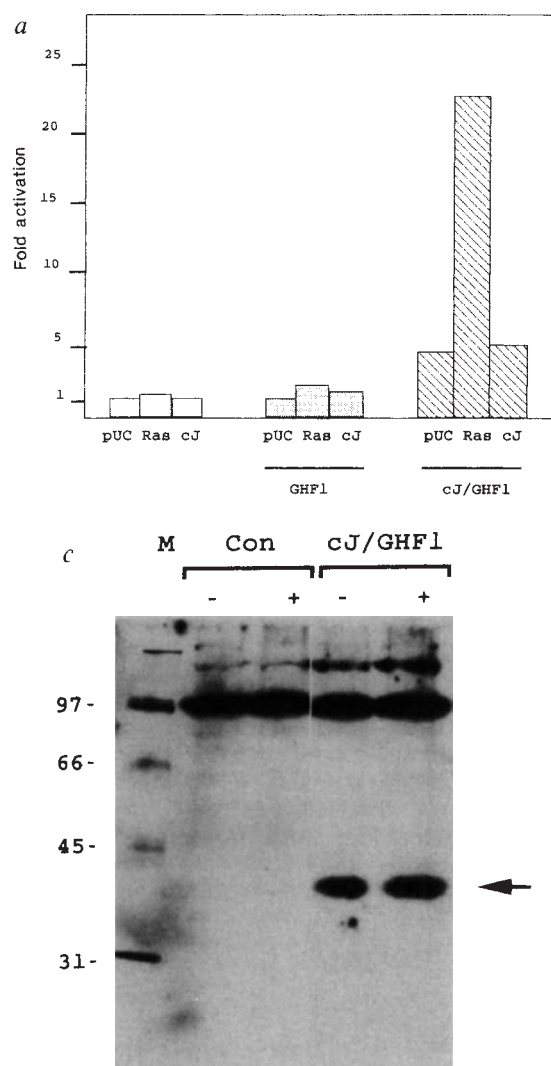


FIG. 5 Ha-Ras stimulates the activity and phosphorylation of the c-Jun activation domain. **a**, NIH3T3 cells were transfected with Jun-GHF1 and GHF-1 expression vectors (5 μ g per plate each) and the -90GHF1-CAT(Δ AP-1) reporter (10 μ g per plate) in the presence (+) or absence (-) of Ha-Ras or c-Jun expression vectors (5 μ g per plate each) as indicated. The cells were collected 24 h after transfection and the levels of CAT activity determined. The values shown are averages of three experiments. **b**, F9 cells were transfected with Jun-GHF1 and GHF-1 expression vectors (7 μ g per plate) in the presence or absence of pZIPneoRas (14 μ g per plate). The cells were labelled 8 h after transfection for 2.5 h with either Trans[³⁵S]label or [³²P]orthophosphate. The synthesis and phosphorylation of Jun-GHF1 and GHF-1 proteins were examined by radioimmunoassay of equal amounts of cell lysates using a GHF-1-specific antipeptide antibody⁴⁸, as described above. **c**, F9 cells were transfected with 10 μ g of either pUC18 (con) or RSV-Jun-GHF1 (J.GHF1) in the presence (+) or absence (-) of 5 μ g pZIPneoRas. Nuclear extracts were prepared 24 h later and analysed for Jun-GHF1 expression using anti-c-Jun antibodies raised against an N-terminal peptide. **d**, The Jun-GHF1 protein was immunoprecipitated from equal numbers of F9 cells transiently transfected with Jun-GHF1 expression

vector in the presence or absence of pZIPneoRas and after SDS-PAGE was eluted and digested with trypsin. Tryptic digests were spotted on thin-layer plates and mapped as described above.

METHODS. The -90GHF1-CAT(Δ AP-1) reporter was derived from -90GHF1-CAT (ref. 38) by deletion of the *Drall-Ndel* fragment of pUC which contains a high-affinity AP-1 site. To analyse the level of Jun-GHF1 expression, nuclear extracts of transfected cells were prepared by established procedures⁴⁹ and 200 μ g extract protein were separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane for 3 h at 4 $^{\circ}$ C at 250 mA in 25 mM Tris, 192 mM glycine and 20% methanol. After blocking in 3% powdered milk, the membranes were incubated with anti-c-Jun antibodies directed against an N-terminal peptide and probed with [¹²⁵I]protein A (Amersham) at 0.4 μ Ci ml⁻¹ as described⁵⁰.

in Jun-GHF1 are identical to the N-terminal sites of c-Jun, x, y and z, as verified by mixing experiments (data not shown). As before, Ha-Ras stimulated phosphorylation of sites x and y without significantly affecting site z (Fig. 5d).

To examine further whether the site of Ha-Ras action is the N-terminal activation domain of c-Jun, we used a c-Jun mutant deleted for its first 87 amino acids. This mutant, c-Jun Δ 1-87, retains only 10% of wild-type activity and was augmented only 1.5-fold by Ha-Ras compared to the fourfold stimulation of wild-type c-Jun (Fig. 6a). Although c-Jun Δ 1-87 was expressed at 50% the level of wild-type c-Jun, it was phosphorylated to a much lesser extent and its phosphorylation was no longer stimulated by Ha-Ras (Fig. 6b). Phosphopeptide maps of c-Jun Δ 1-87 indicated that it was no longer phosphorylated on sites x and y (Fig. 6c). In agreement with the lower level of GSK3 in F9 cells (J. Woodgett, personal communication), the GSK3 cluster was underphosphorylated in these cells and its phosphorylation state was no longer affected by Ha-Ras (compare Figs 6c and 4).

Discussion

Functional cooperation between two different oncogenes, as seen in REF transformation assays, is a well established phenomenon thought to be related to multistep carcinogenesis⁴⁰⁻⁴², but its biochemical basis is not well understood.

Cooperation implies that either the two oncogenes function in complimentary pathways, or that one oncogene enhances the activity of the other⁴². In the case of c-jun and Ha-ras, which cooperate in REF transformation³, the findings presented above are consistent with the action of Ha-Ras and c-Jun in a common pathway in which Ha-Ras affects upstream functions that control c-Jun activity, the downstream effector of this pathway. Stable and transient expression of activated Ha-Ras increases AP-1 activity^{18,20,21}, and although acute expression of Ha-Ras induces both c-fos and c-jun^{23,24}, transient induction of c-fos cannot account for the sustained increase in AP-1 activity seen in stably transformed cells²⁰. In both transactivation or transformation assays, c-Jun is produced from an expression vector whose promoter is not affected by Ha-Ras, and so the effect of Ha-Ras on both functions must be mediated either by stimulation of c-Jun activity or by activation of a different pathway complementing c-Jun action. The experiments described here clearly demonstrate that Ha-Ras stimulates c-Jun activity and that this correlates with increased phosphorylation of c-Jun at two N-terminal sites, x and y.

Several observations suggest that sites x and y are within the c-Jun activation domain and that their increased phosphorylation is intimately associated with the potentiation of c-Jun activity. First, x and y are located within the part of c-Jun used

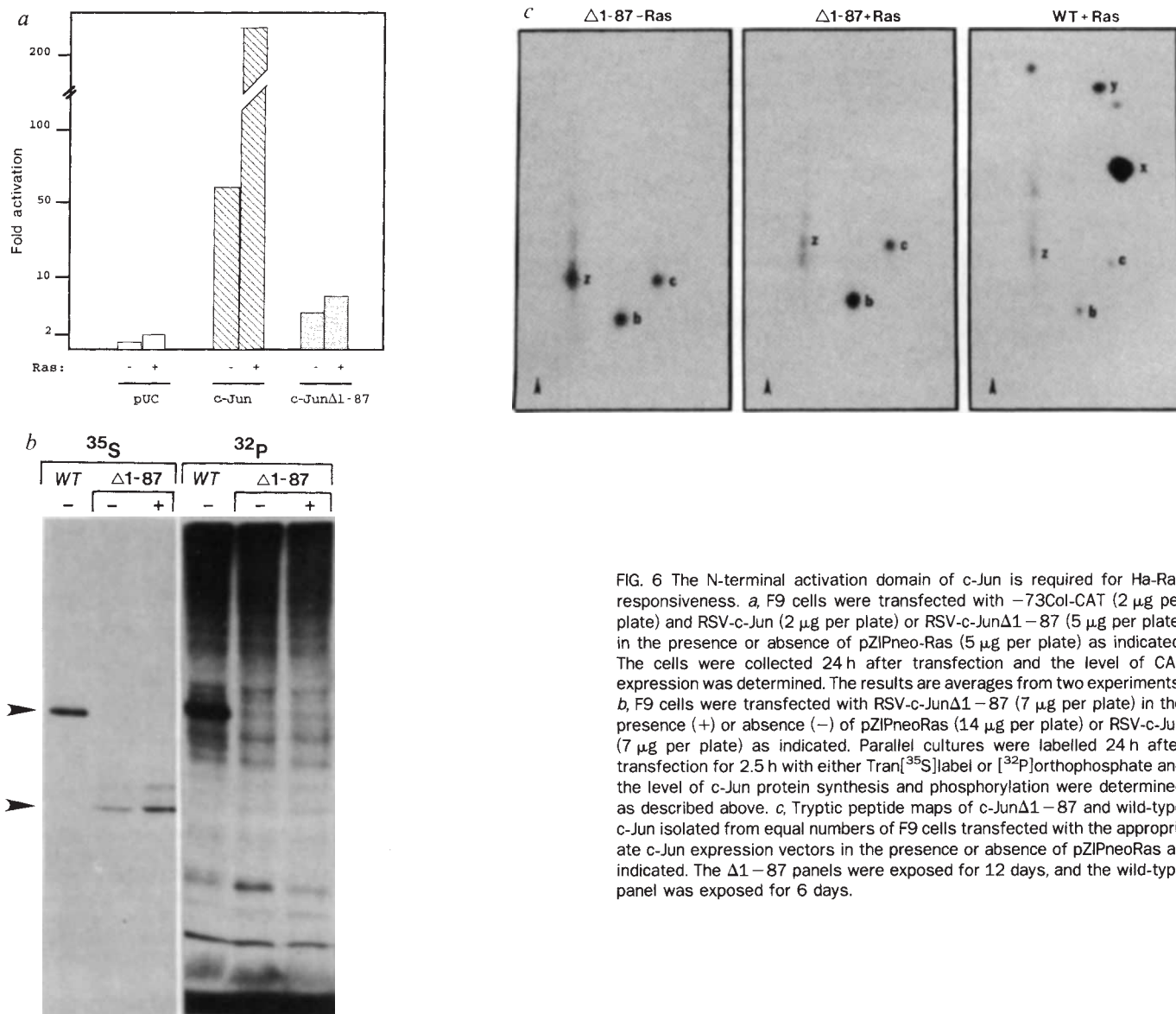


FIG. 6 The N-terminal activation domain of c-Jun is required for Ha-Ras responsiveness. **a**, F9 cells were transfected with -73Col-CAT (2 μ g per plate) and RSV-c-Jun (2 μ g per plate) or RSV-c-Jun Δ 1-87 (5 μ g per plate) in the presence or absence of pZIPneo-Ras (5 μ g per plate) as indicated. The cells were collected 24 h after transfection and the level of CAT expression was determined. The results are averages from two experiments. **b**, F9 cells were transfected with RSV-c-Jun Δ 1-87 (7 μ g per plate) in the presence (+) or absence (-) of pZIPneoRas (14 μ g per plate) or RSV-c-Jun (7 μ g per plate) as indicated. Parallel cultures were labelled 24 h after transfection for 2.5 h with either Tran[³⁵S]label or [³²P]orthophosphate and the level of c-Jun protein synthesis and phosphorylation were determined as described above. **c**, Tryptic peptide maps of c-Jun Δ 1-87 and wild-type c-Jun isolated from equal numbers of F9 cells transfected with the appropriate c-Jun expression vectors in the presence or absence of pZIPneoRas as indicated. The Δ 1-87 panels were exposed for 12 days, and the wild-type panel was exposed for 6 days.

to generate the Ha-Ras-responsive chimaeric activator Jun-GHF1. Second, deletion of the first 87 amino acids of c-Jun results in loss of these sites, decreases its phosphorylation and renders it insensitive to Ha-Ras. Third, the magnitude of the increase of c-Jun phosphorylation is very similar to the increase in its activity. The most parsimonious interpretation of the results is that phosphorylation of c-Jun at sites x and y augments its ability to activate transcription. In this respect c-Jun behaves in a similar way to its relative CREB, whose activity is stimulated by phosphorylation of a specific site within its activation domain by the cyclic AMP-dependent protein kinase⁴³. Other less likely interpretations include an Ha-Ras-stimulated coactivator, or inactivation of a specific repressor that interacts with the c-Jun activation domain. Recent findings have suggested that c-Jun may be regulated by a repressor³⁰, but it is important to realize that evidence supporting its existence can be obtained only in HeLa cells and not in three other mammalian cell lines (F9, NIH3T3 and HepG2) and, most importantly, not in REF cultures. A Ha-Ras-stimulated coactivator is also an unlikely explanation because such a factor is expected to be a pleiotropic regulator and not c-Jun-specific. Yet, previous experiments indicate that Ha-Ras leads to specific activation of the TRE¹⁸⁻²².

Expression of Ha-Ras has several effects on AP-1 activity. Initially it leads to transient induction of *c-fos* and *c-jun*^{23,24} and increased phosphorylation of c-Jun. Elevated *c-jun* expression is maintained by positive autoregulation¹⁶ but *c-fos* expression is transient^{12,24}. Although the effect of Ha-Ras on c-Fos phosphorylation is unknown, a recent report indicates that increased phosphorylation converts c-Fos to an auto-repressor contributing to its transient expression⁴⁴. Therefore, most of the AP-1 activity in Ha-Ras-transformed cells is probably

sustained by a phosphorylated form of c-Jun. This conclusion is supported by the transfection experiments which show that the Ha-Ras effect on c-Jun activity is not a result of c-Fos induction. Phosphorylation of other Jun proteins has not been analysed yet, but it is of interest that JunB is not stimulated by Ha-Ras (Fig. 2) and displays very weak transforming activity in the presence of Ha-Ras⁴⁵.

Regulation of c-Jun activity by phosphorylation is complex. Phosphorylation of c-Jun at sites next to its DNA-binding domain decreases DNA-binding activity and dephosphorylation of one of these sites by a putative PKC-activated phosphatase results in increased DNA-binding activity¹⁷. The present work suggests that phosphorylation of c-Jun at two N-terminal sites increases its transactivating potential. But full activation should depend on dephosphorylation of the inhibitory C-terminal site to allow DNA binding. TPA affects c-Jun DNA binding by inducing dephosphorylation of the C-terminal sites¹⁷, but the effect of Ha-Ras is more complex. In addition to increasing phosphorylation of the N-terminal sites, Ha-Ras affects the state of the C-terminal phosphorylation sites resulting in accumulation of c-Jun forms that are underphosphorylated at this region and can therefore bind to DNA¹⁷. This effect of Ha-Ras is similar to that of TPA and is consistent with its ability to increase phosphatidylinositol turnover and PKC activity⁴⁶. But, the effect of Ha-Ras on the N-terminal sites is unique and could be the result of its PKC-independent action⁴⁷. Although further investigation is required to identify the Ha-Ras-activated c-Jun-kinase, the present work identifies a physiologically relevant substrate (c-Jun) that can be used to search for downstream targets of Ha-Ras action and to provide a biochemical explanation for oncogene cooperation. □

Received 31 December 1990; accepted 3 April 1991.

- Vogt, P. K. & Bos, T. J. *Adv. Cancer Res.* **55**, 1-35 (1990).
- Bos, T. J. *et al. Genes Dev.* **4**, 1677-1687 (1990).
- Schütte, J., Minna, J. & Birrer, M. *Proc. natn. Acad. Sci. U.S.A.* **86**, 2257-2261 (1989).
- Bohmann, D. *et al. Science* **238**, 1386-1392 (1987).
- Angel, P. *et al. Nature* **332**, 166-171 (1988).
- Angel, P. *et al. Cell* **49**, 729-739 (1987).
- Lee, W., Mitchell, P. J. & Tjian, R. *Cell* **49**, 741-752 (1987).
- Karin, M. in *Molecular Aspects of Cellular Regulation* (eds Cohen, P. & Foulkes, G.) **6**, 143-161 (Elsevier/North-Holland Biomedical, Amsterdam, 1990).
- Chiu, R., Angel, P. & Karin, M. *Cell* **59**, 979-986 (1989).
- Hirai, S. I., Ryseck, R. P., Mehta, F., Bravo, R. & Yaniv, M. *EMBO J.* **8**, 1433-1439 (1989).
- Yang-Yen, H.-F., Chiu, R. & Karin, M. *New Biol.* **2**, 351-361 (1990).
- Greenberg, M. E. & Ziff, E. B. *Nature* **311**, 433-438 (1984).
- Quantin, B. & Breathnach, R. *Nature* **334**, 538-539 (1988).
- Ryseck, R. P., Hirai, S. I., Yaniv, M. & Bravo, R. *Nature* **334**, 535-537 (1988).
- Lamph, W. W., Wamsley, P., Sassone-Corsi, P. & Verma, I. M. *Nature* **344**, 629-631 (1988).
- Angel, P., Hattori, K., Smeal, T. & Karin, M. *Cell* **55**, 875-885 (1988).
- Boyle, W. J. *et al. Cell* **64**, 573-584 (1991).
- Imler, J. L., Schatz, C., Wasylyk, C., Chatton, B. & Wasylyk, B. *Nature* **332**, 275-278 (1988).
- Piette, J., Hirai, S. I. & Yaniv, M. *Proc. natn. Acad. Sci. U.S.A.* **85**, 3401-3405 (1988).
- Wasylyk, C., Imler, J. L. & Wasylyk, B. *EMBO J.* **7**, 2475-2483 (1988).
- Schonthal, A., Herrlich, P., Rahmsdorf, H. J. & Ponta, H. *Cell* **54**, 325-334 (1988).
- Herrlich, P. & Ponta, H. *Trend Genet.* **5**, 112-116 (1989).
- Sistonen, L., Holtta, E., Makela, T. P., Keski-Oja, J. & Alitalo, K. *EMBO J.* **8**, 815-822 (1989).
- Stacey, D. W., Watson, T., Kung, H.-F. & Curran, T. *Molec. cell. Biol.* **7**, 523-527 (1987).
- Dotto, G. P., Parada, L. F. & Weinberg, R. A. *Nature* **318**, 472-475 (1985).
- Zarbl, H., Latrelle, J. & Jolicoeur, P. *Cell* **51**, 357-369 (1987).
- Wisdom, R. & Verma, I. M. *Molec. cell. Biol.* **10**, 5626-5633 (1990).
- Land, H. *et al. Molec. cell. Biol.* **6**, 1917-1925 (1988).

- Imler, J. L., Ugarte, E., Wasylyk, C. & Wasylyk, B. *Nucleic Acids Res.* **16**, 3005-3012 (1988).
- Baichwal, V. R. & Tjian, R. *Cell* **65**, 815-823 (1990).
- Angel, P., Smeal, T., Meek, J. & Karin, M. *New Biol.* **1**, 35-43 (1989).
- Jaggi, R., Salmons, B., Muellener, D. & Groner, B. *EMBO J.* **5**, 2609-2616 (1986).
- Jonat, C. *et al. Cell* **62**, 1189-1204 (1990).
- Yang-Yen, H.-F. *et al. Cell* **62**, 1205-1215 (1990).
- Lucibello, F. *et al. EMBO J.* **9**, 2827-2834 (1990).
- Chiu, R. *et al. Cell* **54**, 541-552 (1988).
- Smeal, T., Angel, P., Meek, J. & Karin, M. *Genes Dev.* **3**, 2091-2100 (1989).
- Theill, L., Castrillo, J.-L., Wu, D. & Karin, M. *Nature* **342**, 945-948 (1989).
- McCormick, A., Brady, H., Theill, L. E. & Karin, M. *Nature* **345**, 829-832 (1990).
- Weinberg, R. A. *Cancer Res.* **49**, 3713-3721 (1989).
- Ruley, H. E. *Cancer Cells* **2**, 258-268 (1990).
- Hunter, T. *Cell* **64**, 249-270 (1991).
- Gonzalez, G. A. & Montminy, M. R. *Cell* **59**, 675-680 (1989).
- Ofir, R., Dwarki, V. J., Rashid, D. & Verma, I. M. *Nature* **348**, 78-80 (1990).
- Schutte, J. *et al. Cell* **59**, 987-997 (1989).
- McCormick, F. in *Oncogenes and the Molecular Origins of Cancer* (ed. Weinberg, R. A.) 125-145 (Cold Spring Harbor Laboratory, New York, 1989).
- Lacal, J. C., Fleming, P. T., Warren, B. S., Blumberg, P. S. & Aaronson, S. A. *Molec. Cell. Biol.* **7**, 4146-4149 (1987).
- Bodner, M. *et al. Cell* **55**, 505-518 (1988).
- Radler-Pohl, A., Pfeuffer, I., Karin, M. & Serfling, E. *New Biol.* **2**, 566-573 (1990).
- Harlow, E. & Lane, D. *Antibodies, A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1988).

ACKNOWLEDGEMENTS. We thank T. Hunter for advice, help in peptide mapping and comments on the manuscript and C. Der for expression vectors and cells. B.B. was supported by a Fogarty International Fellowship. This work was supported by the NIH and Council for Tobacco Research.

Test of the decaying dark matter hypothesis using the Hopkins Ultraviolet Telescope

A. F. Davidsen, G. A. Kriss, H. C. Ferguson*,
W. P. Blair, C. W. Bowers, W. V. Dixon,
S. T. Durrance, P. D. Feldman, R. C. Henry,
R. A. Kimble†, J. W. Kruk, Knox S. Long‡,
H. W. Moos & O. Vancura

Center for Astrophysical Sciences, Department of Physics and Astronomy,
The Johns Hopkins University, 34th and Charles Streets, Baltimore,
Maryland 21218, USA

SCIAMA has argued¹⁻⁴ that the dark matter associated with galaxies, clusters of galaxies and the intergalactic medium consists of τ neutrinos of rest mass 28–30 eV, whose decay generates ultraviolet photons of energy $\sim m_\nu/2 \approx 14$ –15 eV. We have carried out a test of this hypothesis using the Hopkins Ultraviolet Telescope, which was flown aboard the space shuttle Columbia as part of the Astro-1 mission in December 1990. A straightforward application of Sciama's model predicts that we should have observed, from the rich galaxy cluster Abell 665, a spectral line from neutrino decay photons with a signal-to-noise ratio of ~ 30 . We detected no such emission. For neutrinos (or any similar dark matter particle) in the mass range 27.2–32.1 eV, our observations set a lower lifetime limit significantly greater than Sciama's model requires.

The idea is based on work by several previous authors⁵⁻⁷, but Sciama's theory significantly narrows the expected energy range of the decay photon and requires a lifetime for the decay of $\tau \approx 1-3 \times 10^{23}$ s, substantially shorter than lifetimes predicted in most standard theories of particle physics⁸, but compatible with some left-right-symmetric models⁹. The severely constrained neutrino mass and lifetime result from Sciama's assumption that the decay photons are responsible for the partial ionization of the H I regions in the galaxy, as well as the ionization of the Lyman α clouds seen in the spectra of quasars at large redshifts. The short lifetime has the added benefit of making the hypothesis eminently testable.

The energy of the decay photons in Sciama's theory is only slightly above the ionization potential of hydrogen, or Lyman limit, at 13.6 eV. Consequently, a cluster of galaxies requires only a modest redshift to make the line emission expected from the decaying dark matter particles longer than the Lyman limit at 912 Å, where the interstellar hydrogen in our Galaxy becomes transparent. The Hopkins Ultraviolet Telescope (HUT) (Davidsen *et al.*, manuscript in preparation) has been optimized for spectroscopic observations in the 912–1,200-Å band (which is inaccessible to other space telescopes except for the low-resolution spectrometer on Voyager), and is therefore ideal for searching for the predicted neutrino decay line in clusters with redshifts $z \leq 0.5$. The full spectral range covered by HUT is 830–1,850 Å in first order. HUT also includes a large aperture (17×116 arcsec) which projects to a substantial volume within a typical cluster and therefore yields a strong signal for the predicted neutrino decay line.

We selected several rich clusters in the range $0.1 \leq z \leq 0.2$ for observation with HUT on the Astro-1 mission, but because of Spacelab system-level problems and a weather-related shortening of the mission, only one of these clusters, Abell 665, was successfully observed.

Abell 665 is the richest cluster in the Abell catalogue¹⁰ and a luminous X-ray source^{11,12}. A recent study¹³ yields $z = 0.18144 \pm 0.00084$ and a velocity dispersion $\sigma = 1,201_{-126}^{+183}$ km s⁻¹ for 33 galaxies that were found to be cluster members. We assume the projected mass density of the cluster, including both the luminous and dark matter, follows a King model¹⁴ of the form $\mu(r) = 2r_c\rho_0(1+r^2/r_c^2)^{-1}$ with a core radius $r_c = 0.5$ Mpc (ref. 15). The central mass density is then $\rho_0 = 1.7 \times 10^8 \sigma^2/r_c^2 M_\odot \text{ Mpc}^{-3} = 1.0 \times 10^{15} M_\odot \text{ Mpc}^{-3}$. The total mass integrated over the large HUT slit, which covered 68×457 kpc at the centre of Abell 665, is $2.9 \times 10^{13} M_\odot$. (We assume $H_0 = 50$ km s⁻¹ Mpc⁻¹ and $q_0 = \frac{1}{2}$ throughout.)

The uncertainties in this mass estimate contribute a large fraction of the uncertainty in our final result. Although Oegerle *et al.*¹³ find no evidence for substructure in their distribution of velocities for the galaxies in Abell 665, two separate dynamical systems with dispersions of 900 km s⁻¹ and mean velocities separated by 400 km s⁻¹ could reproduce their data. This would lower our mass estimate by a factor of two. On the other hand, the central density and the core radius are not tightly constrained by the available data, and the mass enclosed by the HUT slit could well be higher than we have assumed.

If the mass of Abell 665 is predominantly made up of decaying neutrinos, the luminosity of the cluster is $L = 4.0 \times 10^{54} M_{13} \tau_{23}^{-1} (\epsilon/14 \text{ eV})^{-1}$ photons s⁻¹ in a line at energy ϵ , where M_{13} is the total mass in units of $10^{13} M_\odot$, and τ_{23} is the decay lifetime in units of 10^{23} s. The expected width of the decay line is 11 Å (full width at half maximum, FWHM), or 22 pixels, assuming the neutrino decay emission fills the HUT aperture and that the neutrinos have the velocity dispersion quoted above for the cluster. With the mass obtained above, the predicted flux at Earth is $0.089 \tau_{23}^{-1} (\epsilon/14 \text{ eV})^{-1}$ photons cm⁻² s⁻¹. In the relevant energy range HUT has an effective area of 8.0 cm², leading to an expected count rate of 0.24–0.71 counts s⁻¹ for Sciama's proposed range for the lifetime $\tau \approx 1-3 \times 10^{23}$.

Abell 665 was observed with HUT for a total of 1,932 s on 9 December 1990. The aperture was centred at right ascension $\alpha_{1950} = 8$ h 26 min 24.6 s, declination $\delta_{1950} = +66^\circ 00' 36.0''$, which corresponds to the position of the brightest cluster galaxy (W. Oegerle, personal comm.). Beers and Tonry give the median galaxy position¹⁶ as $\alpha_{1950} = 8$ h 26 min 18.7 s, $\delta_{1950} = +65^\circ 59' 58''$, and the centroid from the Imaging Proportional Counter on Einstein as $\alpha_{1950} = 8$ h 26 min 27.7 s, $\delta_{1950} = +66^\circ 01' 07''$. The HUT slit was oriented at a position angle of 40° to include both the X-ray centre and the median galaxy position. Most of the

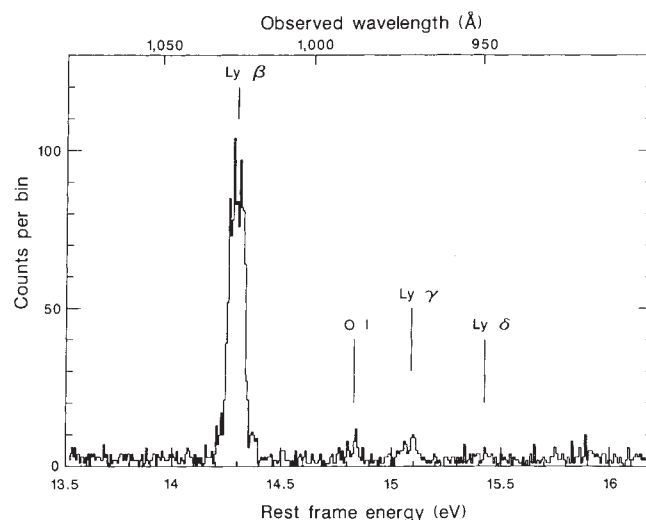


FIG. 1 Portion of the Abell 665 spectrum from the full 1,932-s observation covering the rest-frame energy range 13.6–16.1 eV. The ordinate is in observed counts per 0.51 Å bin. Prominent airglow features observed in other HUT spectra are marked.

* Present address: Institute of Astronomy, University of Cambridge, Madingley Road, Cambridge CB3 0HA, UK.

† Present addresses: Laboratory for Astronomy and Solar Physics, Goddard Space Flight Center, Greenbelt, Maryland 20771, USA (R.A.K.); Space Telescope Science Institute, 3700 San Martin Drive, Baltimore, Maryland 21218, USA (K.S.L.).

observation (1,378 s) was during orbital night, when airglow line emission is a minimum. The spectrum obtained had no features other than those expected from the airglow based on observations of other faint sources and blank fields during the Astro-1 mission. Figure 1 shows the relevant portion of the data from the full 1,932-s observation, corrected to the rest-frame of Abell 665. The positions of the known airglow lines Ly β , O I at 989 Å, Ly γ , and Ly δ are indicated. The observed flux of the Ly β line is 0.58 counts s⁻¹, essentially equal to the flux expected for the neutrino decay line. The background count rate (the apparent continuum in Fig. 1) of 1.2×10^{-3} per pixel per s is consistent with that from other blank sky observations. About one-third of this rate is due to internal detector background from charged particles (as measured with the aperture closed), and two-thirds is from grating-scattered geocoronal Ly α .

Emission lines from an extended source filling the $17'' \times 116''$ slit have a FWHM of 5.5 Å (11 pixels), as measured from other observations of bright airglow lines through this aperture. The Ly β line in Fig. 1 has a FWHM of 5.7 Å. As mentioned previously, emission from decaying neutrinos broadened by the cluster velocity dispersion would produce a line with FWHM 11 Å (22 pixels). At each pixel in the spectrum, we calculate upper limits to the intensity of an unresolved spectral feature at that location by computing the flux above the background in a surrounding 19-pixel region. This choice of size maximizes the signal-to-noise ratio for detecting a weak feature above the background and is conservative, as colder neutrinos would produce a narrower line which would be easier to detect. The 2σ upper limit is then $[\sum (C_i - B) + 2\sqrt{\sum C_i}]/0.71t$, where B is the background count per pixel, C_i is the number of counts in pixel i , t is the total integration time, and the factor 0.71 is the fraction of the line flux contained in the 19-pixel region. Because this sum averages ~ 45 counts, the Poisson count distribution closely resembles a gaussian in its statistical properties, and we have simply used a factor of twice the Poisson standard deviation.

The only spectral features that exceed these upper limits during the orbital night portion of the observation are Ly β and Ly γ . In the full observation shown in Fig. 1, airglow features due to O I at 989 Å and Ly δ are also visible, and to be conservative we have used the higher fluxes seen for the whole observation in computing our upper limits. We have not yet developed a detailed model of the expected intensity of airglow features in the HUT spectra for the many relevant parameters, such as orbital position and viewing angle relative to the Earth limb and the Sun. Emission from decaying neutrinos could (fortuitously) lie at the same wavelength as Ly β or Ly γ , but as none of the airglow features are broader than the instrumental response, the dispersion of the decaying neutrinos in the cluster would have to be much less than the observed cluster velocity dispersion.

The Ly β line is bright enough that we can set a more stringent limit using the shape of the line profile and the Ly β /Ly α ratio from other observations. To be conservative, we compute the expected Ly β flux using the lowest observed ratio of Ly β /Ly α in the airglow observed by HUT. This comes from an observation of the Crab Nebula. (The Crab was within a few degrees of the anti-solar position on the sky, and the viewing angle during orbital night was almost entirely in the Earth's shadow.) We therefore presume that any contribution from decaying neutrinos to the observed Ly β in our observation of Abell 665 can be at most the amount by which the observed flux exceeds this minimum. Within three pixels of the centre of the Ly β line, we determine our upper limit by subtracting the observed Ly β /Ly α ratio of $(2.47 \pm 0.23) \times 10^{-3}$ in the orbital night portion of the Crab Nebula spectrum from the ratio $(4.03 \pm 0.16) \times 10^{-3}$ observed during orbital night on Abell 665. Scaling this to the Ly α intensity of 116 counts s⁻¹ during orbital night in the Abell 665 observation, we obtain a maximum contribution to the Ly β intensity of 0.181 ± 0.033 counts s⁻¹. We use 0.247 counts s⁻¹ as

our 2σ upper limit within three pixels of the Ly β line centre. The Ly β /Ly α ratio observed in the Abell 665 spectrum is typical of that seen in other orbital night observations of the airglow. We may be able to reduce the upper limits at the Ly β line centre as we develop our understanding of the airglow spectrum.

To eliminate the possibility that the decay line could be hiding in the wings of geocoronal Ly β , we compare the Ly β line profile in the Abell 665 spectrum with that observed in other night observations of weak sources. Using a Ly β line profile obtained from observations of the Crab Nebula and the Perseus cluster, summed and smoothed with a three-point boxcar, we fit a 120-pixel region centred on Ly β in the Abell 665 spectrum, allowing the line centre, its intensity and a constant background to vary freely. The resulting $\chi^2 = 126.40$ indicates excellent agreement between the profiles of the template and the Ly β line of Abell 665. The shift in wavelength between the two lines is less than one pixel. We then add a second emission line at a fixed offset which is an integral number of pixels from the centre of the Ly β emission line. The intensity of the second emission line is increased until $\Delta\chi^2 = 4.2$ (95.4% confidence for one interesting parameter¹⁷) while all other parameters are re-optimized. This sets our 2σ upper limit in the regions 2–11 pixels above and below the centre of the Ly β emission line.

We have converted our upper limits on the decay-line intensity for Abell 665 to a lower limit on the lifetime of the neutrino, taking into account the variation in HUT sensitivity with energy (known to $\pm 20\%$ over the wavelength range of interest from laboratory calibrations and in-flight comparison of several white dwarf spectra to theoretical models; P. Bergeron, personal communication) and galactic extinction of $E_{B-V} = 0.034$ for a galactic neutral hydrogen column density^{18,19} of 4.7×10^{20} cm⁻². We use the extinction law produced by Longo *et al.*²⁰ from an earlier review²¹. Figure 2 shows the result, along with the range of lifetimes and energies predicted by Sciama's theory. Over most of the range of interest the observational limit on the lifetime exceeds 3×10^{24} s, a factor at least 10 times the value required by the decaying dark matter hypothesis. However, a narrow region of parameter space at $E = 14.30 \pm 0.02$ eV, where interference from geocoronal Ly β is severe, would allow the theory to survive, if we have been so unfortunate as to choose a cluster whose redshift hides the neutrino decay line behind geocoronal Ly β . (The error in E is dominated by the uncertainty in the redshift of the cluster.) We expect that detailed analysis of the

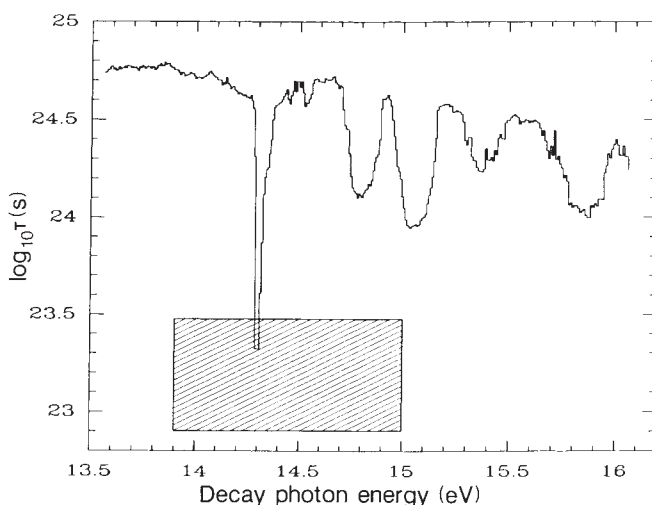


FIG. 2 Logarithm of the 2σ lower limits derived from HUT observations for the lifetime of decaying neutrinos (in s) as a function of the decay photon energy (in eV). The shaded region illustrates the range of energies and lifetimes compatible with Sciama's theory.

airglow data obtained by HUT throughout the Astro-I mission, together with further modelling, will allow us to strengthen our limit in the vicinity of Lyman β .

Highly sensitive observations of the cluster Abell 665 with the HUT thus fail to support the decaying dark matter hypothesis. The theory can survive only under one of the two following conditions: (1) the cluster is several times less massive than we estimate and the redshifted decay photon energy happens to coincide with the Ly β airglow line, or (2) there is

substantial absorption of the decay line by previously unsuspected material along the line of sight. The latter has in fact been suggested by Sciama (personal communication) in response to our result. We believe we can rule out this possibility, but a discussion must be postponed to a future paper (G.A.K., A.F.D. and H.C.F., in preparation). Although neutrinos may yet provide the missing mass, they probably do not decay at rates high enough to explain the ionization balance in the interstellar or intergalactic medium. $\square$

Received 1 February; accepted 2 April 1991.

1. Sciama, D. W. *Astrophys. J.* **364**, 549–554 (1990).
2. Sciama, D. W. *Phys. Rev. Lett.* **65**, 2839–2841 (1990).
3. Sciama, D. W. *Nature* **348**, 617–618 (1990).
4. Sciama, D. W. *Astrophys. J.* **367**, L39–L41 (1991).
5. Cowsik, R. *Phys. Rev. Lett.* **39**, 784–787 (1977).
6. De Rújula, A. & Glashow, S. *Phys. Rev. Lett.* **45**, 942–944 (1980).
7. Melott, A. L. & Sciama, D. W. *Phys. Rev. Lett.* **46**, 1369–1371 (1981).
8. Pal, P. B. & Wolfenstein, L. *Phys. Rev. D* **25**, 766–773 (1982).
9. Maalampi, J. & Roos, M. *Phys. Letters B* **229**, 388–390 (1989).
10. Abell, G. O. *Astrophys. J. Suppl. Ser.* **3**, 211 (1958).
11. Johnson, M. W., Crudace, R. G., Ulmer, M. P., Kowalski, M. P. & Wood, K. S. *Astrophys. J.* **266**, 425–445 (1983).
12. Soltan, A. & Henry, J. P. *Astrophys. J.* **271**, 442–445 (1983).

13. Oegerle, W., Fitchett, M. J., Hill, J. M. & Hintzen, P. *Astrophys. J.* (in the press).
14. Rood, H. J., Page, T. L., Kintner, E. C. & King, I. R. *Astrophys. J.* **175**, 627–647 (1972).
15. Dressler, A. *Astrophys. J.* **226**, 55–60 (1978).
16. Beers, T. C. & Tonry, J. L. *Astrophys. J.* **300**, 557–567 (1986).
17. Avni, Y. *Astrophys. J.* **210**, 642–646 (1976).
18. Burstein, D. & Heiles, C. *Astrophys. J.* **225**, 40–55 (1978).
19. Heiles, C. *Astr. Astrophys. Suppl.* **20**, 37–55 (1976).
20. Longo, R., Stalio, R., Polidan, R. S. & Rossi, L. *Astrophys. J.* **339**, 474–478 (1989).
21. Savage, B. D. & Mathis, J. H. *Ann. Rev. Astr. Astrophys.* **17**, 73–111 (1979).

ACKNOWLEDGEMENTS. We thank L. Madansky, R. Lucas, P. Bergeron and W. Oegerle for information, D. Sciama for discussions, and our colleagues on the HUT team, particularly G. Fountain, B. Ballard, J. Hayes, K. Heffernan, S. Conard, R. Barkhouser and M. Romelfanger, as well as the NASA personnel who helped make the Astro-1 Mission successful. This work was supported by NASA.

Detection of binaries in the core of the globular cluster M15 using calcium emission lines

Brian W. Murphy*, René G. M. Rutten†, Paul J. Callanan‡, Patrick Seitzer§, Philip A. Charles¶||, Haldan N. Cohn¶ & Phyllis M. Lugger¶

* Institute of Astronomy, University of Utrecht, Postbus 80.000, 3508 TA Utrecht, The Netherlands

† Astronomical Institute "Anton Pannekoek", Kruislaan 403, 1098 SJ Amsterdam, The Netherlands

‡ Department of Astrophysics, Oxford University, Keble Road, Oxford OX1 3RH, UK

§ Space Telescope Science Institute, 3700 San Martin Drive, Baltimore, Maryland 21218, USA

|| Observatorio del Roque de los Muchachos, Apt. 303, Santa Cruz de La Palma, Tenerife, Canary Islands

¶ Department of Astronomy, Indiana University, Bloomington, Indiana 47405, USA

M15 is the prototypical collapsed-core globular cluster. Having undergone collapse, its core is believed now to be expanding, with energy for the re-expansion provided by binary stars, which turn gravitational potential energy into kinetic energy¹. Because these binary stars are generally more massive than single stars, they will have settled to the centre of the cluster². We report here that several of the stars at the core of M15 show Ca II H- and K-line emission, characteristic of young, rapidly rotating stars and close binaries³. We argue that the emission from M15 comes from primordial binaries, in which a period of spin-up has led to magnetic field generation by enhanced dynamo action, which in turn causes heating of the stellar chromospheres. If this interpretation is correct, the Ca H and K emission may provide an important diagnostic tool of the binary population in cluster cores, and thus of the cluster dynamics.

Our observations were taken at the Isaac Newton Group of Telescopes on La Palma using the 4.2-m William Herschel telescope, equipped with the ISIS triple spectrograph, on the nights of 8, 11 and 13 August 1990. The blue camera and CCD-IPCS (charge-coupled device-image photon counting system) in combination with the H2400B grating gave us a dispersion of 8 Å mm⁻¹ and a resolution of 0.4 Å (5 pixels full width half maximum, FWHM). Spatial sampling along the slit was

0.3" per pixel, the final resolution at the detector was 1.1" FWHM. Although we did not spatially resolve the three stars in the core, AC214, 215, and 216 (ref. 4), as shown in the contour plot in Fig. 1 from a blue Canada–France–Hawaii telescope CCD image taken in 0.4" seeing (P.M.L. *et al.*, manuscript in preparation), the light at these wavelengths is dominated by the bluest objects. The seeing on the night of 8 August was 1.2" to 1.5". On the nights of 11 and 13 August the seeing was better than 0.7" FWHM as measured on the spectrograph slit jaws.

Figure 2a shows a 40-min spectrum of a 3.0" × 1.0" area centred on the bluest of three stars in the central core: AC214 ($V = 14.75$, $B - V = 0.10$ (ref. 4)). Figure 2b shows the spectrum of the star AC623 ($V = 13.50$, $B - V = 1.04$ (ref. 4)), which is located 16" from the centre. In both cases emission in the cores of the Ca II

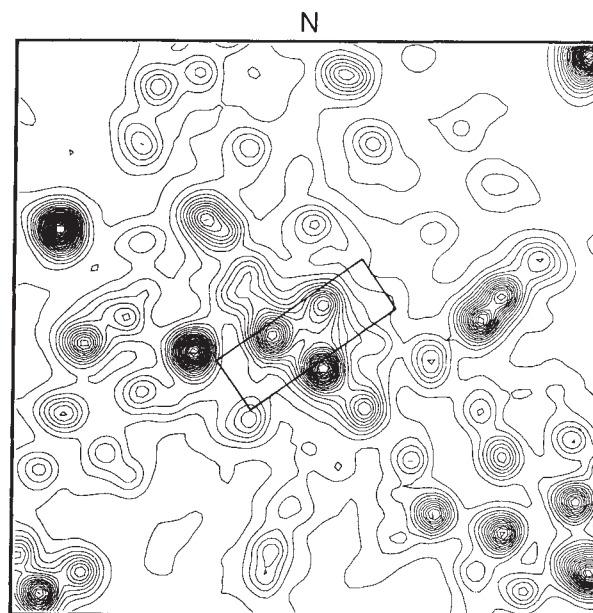


FIG. 1 A B-band image of the central 10" × 10" of M15 taken in September 1989 using the image-stabilization, high-resolution camera on the Canada–France–Hawaii telescope (P.M.L. *et al.*, manuscript in preparation). The pixel size is 0.11". The box in the centre of the diagram shows the position of the slit for the spectra in Fig. 2a.

H and K lines is clearly detected. This line-core emission is produced by heating of the outer atmosphere to chromospheric temperature. The heating is controlled by the magnetic field created by the star's rapid rotation^{3,5}. As a comparison the Sun, a moderately active star, only exhibits emission peaks up to a few per cent of its continuum level in surface-integrated light. Stars with emission peaks much larger than those shown in Fig. 2a and b do exist as well. FK Comae is an example of a giant exhibiting strong emission; it has a rotation period of 2.4 days⁶. It is believed that FK Comae is either a single star that coalesced from a close binary⁷ or a close binary with a secondary star of low mass⁸. We have also included a spectrum of the star AC9 ($V = 14.16$, $B - V = 1.16$ (ref. 4)), located $\sim 15''$ from the centre of the cluster. This spectrum, shown in Fig. 2c, shows little or no emission. As the stars in M15 are ~ 15 Gyr old, magnetic braking should have reduced their zero-age main-sequence rotation rates sufficiently for little magnetic activity to occur. Two mechanisms that could reactivate old stars are (1) tidal capture in a close binary system and subsequent spin-up of the stars and (2) the existence and hardening of a large population of primordial binaries.

At the time of core collapse a globular cluster such as M15 is predicted to have a core density that may exceed $10^8 M_{\odot} \text{pc}^{-3}$ (ref. 9). At these high densities stars can no longer be assumed to act dynamically as point masses. It is likely that tidal interactions will occur, causing hard binaries to form (binaries with internal orbital velocities exceeding the local stellar velocity dispersion). When a main-sequence star is captured by a neutron star, its semi-major axis will be $\leq 3R_*$, where R_* is the radius of the main-sequence star. At these small distances tidal forces will cause the binary to synchronize stellar rotation with orbital motion, which results in the rotation rate of the star increasing from an almost negligible value to about once a day. This spin-up will promote the generation of magnetic fields by the dynamo action in the stellar interior, which will then result in heating of the outer atmosphere and emission in the Ca II H and K line cores. Evolving models using identical stars¹⁰ give the number of tidally formed binaries near the time of core collapse as $\sim 10^3$. But calculations including both a degenerate component and a main-sequence star component show that the number of tidally formed binaries is ~ 30 near the time of core collapse¹¹. Because we only observed post-main-sequence stars, the emission is unlikely to have been from tidally captured giants, as recent results¹² suggest that a giant's envelope would probably be completely disrupted in a close encounter with a degenerate star. Because it seems unlikely that sufficient numbers of giant-containing binaries could be produced by tidal capture we will not discuss this possibility further.

We now consider the possibility of primordial binaries. Recent evidence^{13,14} indicates that as many as 10% of stars by number in globular clusters could be in the form of binaries. If this estimate is accurate then most of these are probably primordial binaries^{13,14}. These binaries would have segregated to the core of the cluster fairly rapidly as they are heavier on average than typical single stars. After segregation, the central density of binaries will be five to ten times that of singles². These binaries will harden because of three-body encounters with single stars, or they may strongly interact with one another, causing some to be ejected from the core and others to be completely destroyed. But overall, the remaining binary population will gradually harden², and the average orbital period (and hence the rotation period) will therefore decrease. Of course there will still be a large spread of the semi-major axes, but the average should lie between 0.2 and 0.5 AU¹⁵. This would reduce the periods of a large fraction of the binaries to small enough values to cause magnetic activity to commence, producing the observed Ca II H and K. The half-mass radius of the distribution of this binary population would be small², < 0.5 pc. Thus, there will be nearly 15,000 binaries within 0.5 pc of the centre for a cluster of $6 \times 10^5 M_{\odot}$. A conservative estimate is that 10% (1,500

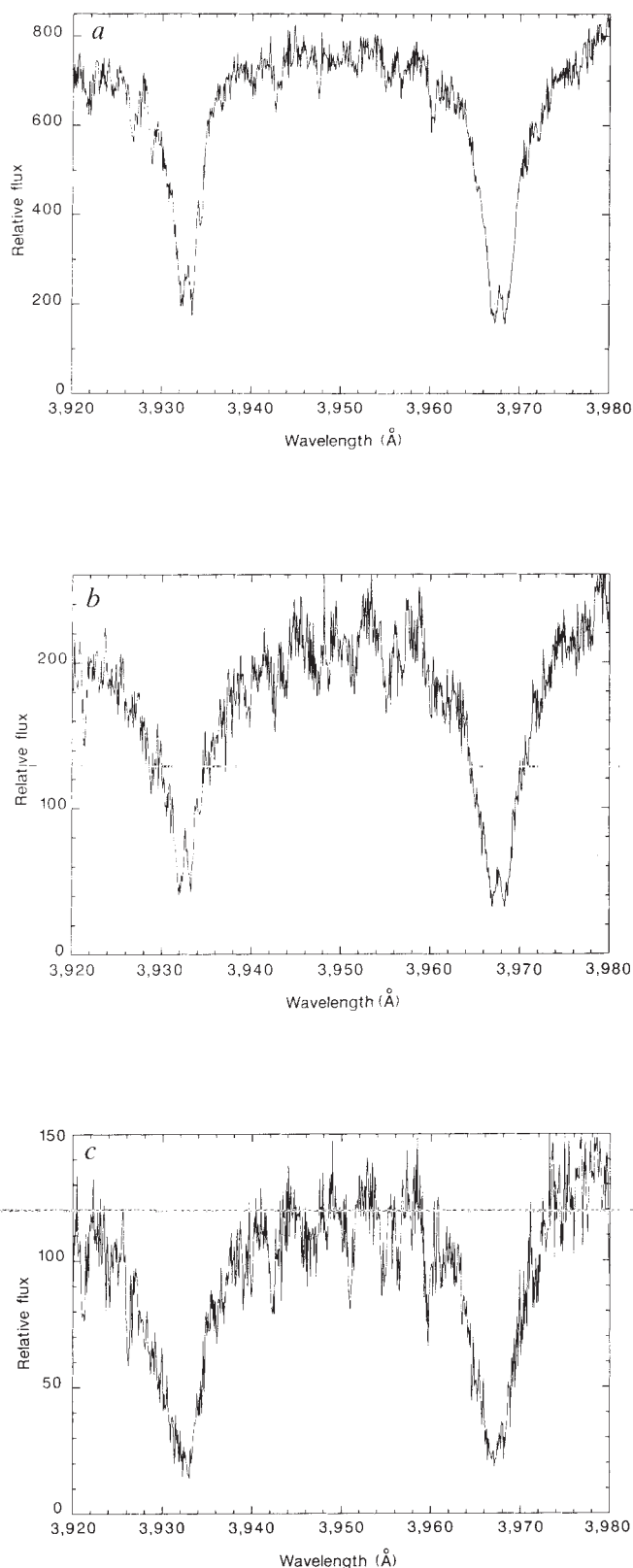


FIG. 2 Spectra taken with the William Herschel telescope. *a*, A $3.0'' \times 1.0''$ region (rectangle in Fig. 1) of the collapsed-core globular cluster M15. *b*, The star AC623 located $16''$ from the centre of M15. Note the emission in the cores of the Ca II H and K lines. This emission rises to a peak of $\sim 40\%$ of the continuum. For comparison, the Sun exhibits emission peaks of Ca II H and K that are $< 5\%$ of the continuum. This unexpected discovery from our August 1990 run may indicate the presence of binaries. *c*, The star AC9 located $15''$ from the centre of M15. The line-core emission, if present, is much weaker than in the spectrum of Fig. 2a and b.

binaries) would show magnetic activity. Many of these binaries are likely to exchange their less massive components for heavier stars such as neutron stars, eventually producing low-mass X-ray binaries and millisecond pulsars¹⁵.

Another mechanism to explain the Ca II H and K emission may be the dissipation of acoustic waves in the atmosphere of red giants or photospheric pulsations of stars on the giant branch^{16–18}. This would also heat the atmosphere of the star, but is most likely to occur for stars near the tip of the red giant branch, and these stars would probably show significant mass loss. Two red giants in the globular cluster NGC6752 show Ca II K emission, and it has been suggested¹⁹ that this is a pulsation instability producing shocks that lead to emission. One problem with this suggestion is that these stars would have radii of $\sim 100 R_{\odot}$. If primordial binaries exist, then giants of this radius could not exist because they would be too close to their companion star. This may in fact explain why M15 and other collapsed-core clusters seem to be bluer in their cores than the surrounding area^{20–22}. If stars near the main-sequence turn-off are prevented from becoming giants because they are in a close binary system, then red giants would be preferentially depleted in the core.

We conclude that the emission in the line cores of Ca II H and K lines that we found in many of the core stars of M15 may indicate the presence of synchronized close binary stars. If binary activity is the source of the emission, then it is likely that the stars are primordial binaries and not tidal-capture binaries. Because primordial binaries are expected to be more concentrated toward the core of the cluster, a radial dependence is expected in the ratio of stars showing Ca II H and K emission to those that do not. Previous searches for binaries in globular clusters have examined giants for radial velocity variations over extended periods¹⁴. Using Ca II H and K emission lines avoids this drawback because we can detect several close binaries in one observation. In future observations we plan to examine the radial dependence of this emission and conduct a survey of other globular clusters. If our interpretation of the emission as a result of binary activity is confirmed, spatially resolved, moderate-resolution spectroscopy could be a new diagnostic tool for determining the presence, distribution and relative numbers of binaries in globular clusters. □

Cross-membrane coupling of chemical spatiotemporal patterns

David Winston, Mukesh Arora, Jerzy Maselko, Vilmos Gáspár & Kenneth Showalter*

Department of Chemistry, West Virginia University, Morgantown, West Virginia 26506-6045, USA

* To whom correspondence should be addressed

CHEMICAL systems may communicate by exchange of common species through mass transport, and such coupling may give rise to dynamical complexity beyond that possible in the independent systems^{1–5}. We report here on dynamical behaviour arising from the diffusive coupling of chemical spatiotemporal patterns across a membrane. Chemical waves appear on Nafion membranes that are loaded with ferroin catalyst and bathed in a mixture of the reagents of the Belousov–Zhabotinsky oscillatory reaction. The waves on each side of the membrane couple by diffusive transport through the membrane. The coupling initially gives rise to the spontaneous appearance of spiral waves, and subsequent behaviour reveals several distinct phases of evolution, ultimately leading to complete spatiotemporal entrainment.

Figure 1a shows an image of regular wave behaviour on the ferroin-loaded Nafion membrane; the three horizontal lines correspond to grey-level profiles depicted in Fig. 2. Arcs defined by the intersection of these lines with a particular wave allow

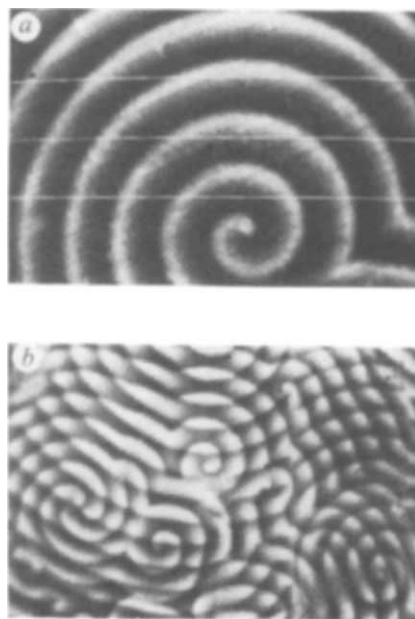


FIG. 1 Chemical waves propagating on the surface of a ferroin-loaded Nafion membrane suspended in a continuous-flow stirred-tank reactor (CSTR) (stirring rate 300 r.p.m.) pumped with Belousov–Zhabotinsky reaction mixture. These digital images^{11,16} show the intensity of light (wavelength 500 nm) transmitted through a membrane 0.18 mm in thickness; field of view is 6.2×4.7 mm. The three horizontal lines correspond to the grey-level profiles in Fig. 2. Reactant concentrations in CSTR: $[\text{NaBrO}_3] = 0.19$ M, $[\text{H}_2\text{SO}_4] = 0.24$ M, $[\text{malonic acid}] = 1.9 \times 10^{-2}$ M, $[\text{ferroin}] = 5.8 \times 10^{-5}$ M, and $[\text{KBr}] = 8.7 \times 10^{-2}$ M; reactor residence time is 56.93 min; temperature maintained at 25.0 ± 0.2 °C. a, Nafion membrane loaded with ferroin to 16.7% capacity, giving strong coupling. The image was obtained 4.0 h after the reaction was initiated. b, Nafion loaded to 38.7% capacity, giving weak coupling. Image obtained 4.5 h after initiating the reaction. Note that the left-most spiral source is a spontaneous three-armed vortex.

Received 29 November 1990; accepted 13 March 1991.

- Goodman, J. *Dynamics of Dense Stellar Systems* (ed Merritt, D.) (Cambridge University Press, 1989).
- Gao, B., Goodman, J., Cohn, H. & Murphy, B. *Astrophys. J.* **370**, 567–582 (1991).
- Zwaan, C. In *Mechanisms of Chromospheric and Coronal Heating* (eds Ulmschneider, P., Priest, E. & Rosner, R.) (Springer, Heidelberg, 1991).
- Aurière, M. & Cordoni, J.-P. *Astr. Astrophys. Suppl. Ser.* **46**, 347–354 (1981).
- Rutten, R. G. M. *Astr. Astrophys.* **177**, 131–142 (1987).
- Herbig, G. *Astrophys. J.* **128**, 259–266 (1958).
- Bopp, B. W. & Stencel, P. E. *Astrophys. J.* **247**, L131–L134 (1981).
- Walter, F. M. & Basri, G. S. *Astrophys. J.* **260**, 735–743 (1982).
- Murphy, B. W., Cohn, H. N. & Hut, P. *Mon. Not. R. astr. Soc.* **245**, 335–349 (1990).
- Statler, T. S., Ostriker, J. P. & Cohn, H. N. *Astrophys. J.* **316**, 626–659 (1987).
- Lee, H. M. *Astrophys. J.* **319**, 772–800 (1987).
- Rasio, F. A. & Shapiro, S. L. *Astrophys. J.* (in the press).
- Latham, D. W., Hazen-Liller, M. L. & Pryor, C. P. in *Stellar Radial Velocities*, IAU Colloq. No. 88 (eds Philip, A. G. D. & Latham, D.) (Reidel, Dordrecht, 1985).
- Pryor, C., McClure, R. D., Fletcher, J. M. & Hesser, J. E. in *Dynamics of Dense Stellar Systems* (ed Merritt, D.) (Cambridge University Press, UK, 1989).
- Hut, P., Murphy, B. W. & Verbunt, F. *Astr. Astrophys.* **241**, 137–141 (1991).
- Pijpers, F. P. & Habing, H. J. *Astr. Astrophys.* **215**, 334–346 (1989).
- Ulmschneider, P. *Astr. Astrophys.* **222**, 171–178 (1989).
- Bowen, G. H. *Astrophys. J.* **329**, 299–317 (1988).
- Dupree, A. K. et al. *Astrophys. J.* **361**, L9–L13 (1990).
- Cederblom, S. E. et al. in *The Formation and Evolution of Star Clusters* (ed. Janes, K.) (P.A.S.P. Conference Series, in the press).
- Djorgovski, S., Piotto, G. & Mallen-Ornelas, G. in *The Formation and Evolution of Star Clusters* (ed. Janes, K.) (P.A.S.P. Conference Series, in the press).
- Djorgovski, S., Piotto, G., Phinney, E. S. & Chernoff, D. *Astrophys. J.* (in the press).

ACKNOWLEDGEMENTS. We thank C. Zwaan for stimulating discussions and advice throughout this project. R.G.M.R. acknowledges support by the Netherlands Foundation for Astronomical Research, with financial aid from the Netherlands Organization for the Advancement of Pure Research. This work is based on observations made with the William Herschel telescope operated on La Palma by the Royal Greenwich Observatory in the Spanish Observatorio del Roque de los Muchachos of the Instituto de Astrofísica de Canarias.

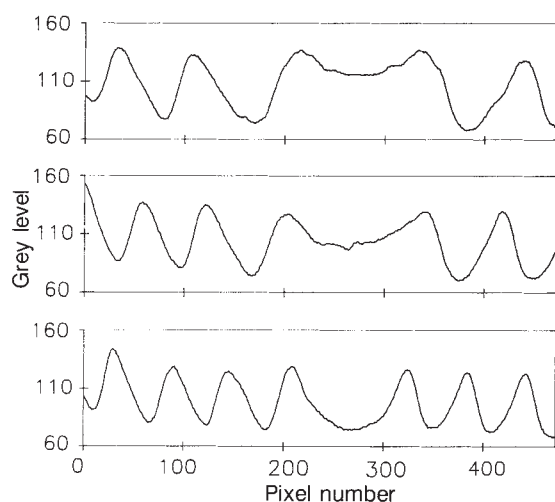


FIG. 2 Grey level as a function of distance for profiles corresponding to the three horizontal lines in Fig. 1a. Each pixel represents 12.1 μm .

calculation of the velocity normal to the front. Plots of wavefront position as a function of time are highly linear (a normal velocity of $0.163 \pm 0.001 \text{ mm min}^{-1}$ was calculated from Fig. 2 and subsequent profiles). These waves are much like those exhibited in thin films of Belousov-Zhabotinsky (BZ) solutions⁶⁻⁸, but with shorter wavelengths and smaller propagation velocities.

The image in Fig. 1b shows a very different type of wave behaviour. Although successive images seem to suggest that the overall behaviour results from two sets of wave patterns evolving relatively independently, closer inspection shows that the two patterns are not independent. Their interaction is sufficiently weak, however, for the features to remain much the same for many hours.

The behaviour in Fig. 1b can be understood by considering the characteristics of Nafion, a perfluorosulphonic acid ionomer^{9,10}. Partial ionization of covalently bound sulphonic acid groups gives rise to an overall net negative charge on the membrane, and negative ions do not significantly penetrate the resin matrix. The chemical-wave activity is therefore confined to the membrane surface because the anion BrO_3^- is an essential reactant. Studies of BZ chemical waves on polystyrene cation-

exchange beads have clearly demonstrated that wave activity occurs on the surface of the resin matrix¹¹. These considerations indicate that the waves in Fig. 1a also occur on both sides of the membrane; here, however, it is apparent that the wave activity is synchronized. The different behaviour in Fig. 1a and b results from strong coupling across the membrane in a and weak coupling in b.

The best candidate for the messenger species that mediates the coupling is bromous acid, the essential autocatalyst of the BZ reaction⁸. Bromous acid is a neutral molecule in the acidic reaction mixture and can diffuse readily through the membrane. A critical factor in the effectiveness of the messenger is the extent of ferroin loading: the entrained waves in Fig. 1a occur on a membrane in which 16.7% of the cation-exchange sites are occupied by ferroin; the relatively uncoupled waves in Fig. 1b occur on a membrane loaded to 38.7%. Higher loading apparently results in the interception of HBrO_2 in an oxidation-reduction reaction with ferroin¹², although other oxybromine species, such as BrO_2 , might also serve as messengers and be similarly intercepted. Alternatively, it is possible that the diffusive transport of the messenger species is simply impeded by the bulky ferroin complex ions in the channels of the membrane.

The images in Fig. 3 show a system in which the wave behaviour is coupled over the course of many hours. Near the beginning of the experiment (Fig. 3a), faint semicircular patterns appear on each side of the membrane. The effects of cross-membrane coupling are clearly evident: spiral waves are spontaneously initiated at crossing points of the superimposed waves. The complex pattern of strongly coupled waves in Fig. 3b appears 1.0 h later. The pattern evolves to that shown in Fig. 3c 4.5 h later, with entrained but irregular behaviour displacing the cross-hatched patterns. The image in Fig. 3d shows complete entrainment and regular behaviour 31 h after initiation of the reaction.

These results can be viewed as the spatiotemporal analogue of coupled chemical oscillators^{4,5}. In the strongly coupled system (Fig. 1a), the waves on each side of the membrane are entrained throughout. With weaker coupling (Fig. 1b), the patterns develop differently on each side of the membrane and seem at first to be independent, but become gradually entrained over long timescales (Fig. 3). Insights into the mechanism of entrainment may be obtained using a four-variable model based on two diffusively coupled two-dimensional chemical-wave systems. We use a computationally efficient scheme for excitable

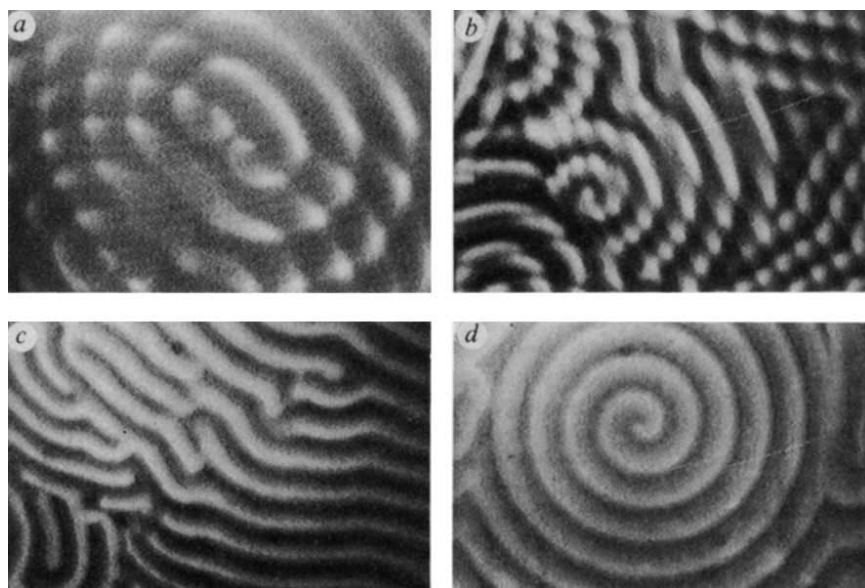


FIG. 3 Transition of spatiotemporal patterns from uncoupled to entrained; conditions are the same as in Fig. 1b. a, Behaviour after 5.5 h; b, 6.5 h; c, 11.0 h; d, 31.1 h. (The image of the spiral source in d was taken at a location near to that of the earlier images.)

media¹³, which mimics the fast-slow dynamics of the Belousov-Zhabotinsky reaction¹⁴. Figure 4 shows one spiral on each side of the model membrane, evolving from uncoupled in *a* to fully entrained, although somewhat irregular, behaviour in *c*. An identical calculation with slightly higher coupling strength yields the more ordered behaviour in *d*, dominated by a single entrained spiral. Intermediate behaviour in *b* demonstrates the importance of relative wave orientation in the coupling, with ray-like domains of relatively uncoupled behaviour, and of

partial and complete entrainment. The initially uncoupled spirals exhibit slightly different rotational periods (in a ratio of 11:12), and the higher-frequency spiral dominates throughout the evolution. The period of the entrained spiral in Fig. 4*d* lies between that of the initial spirals, reflecting the characteristics of both systems. These results indicate that the patterns observed in the experimental case result from spirals with slightly different frequencies on each side of the membrane. Other calculations for rotational periods in the ratio 1:2 show phase locking with

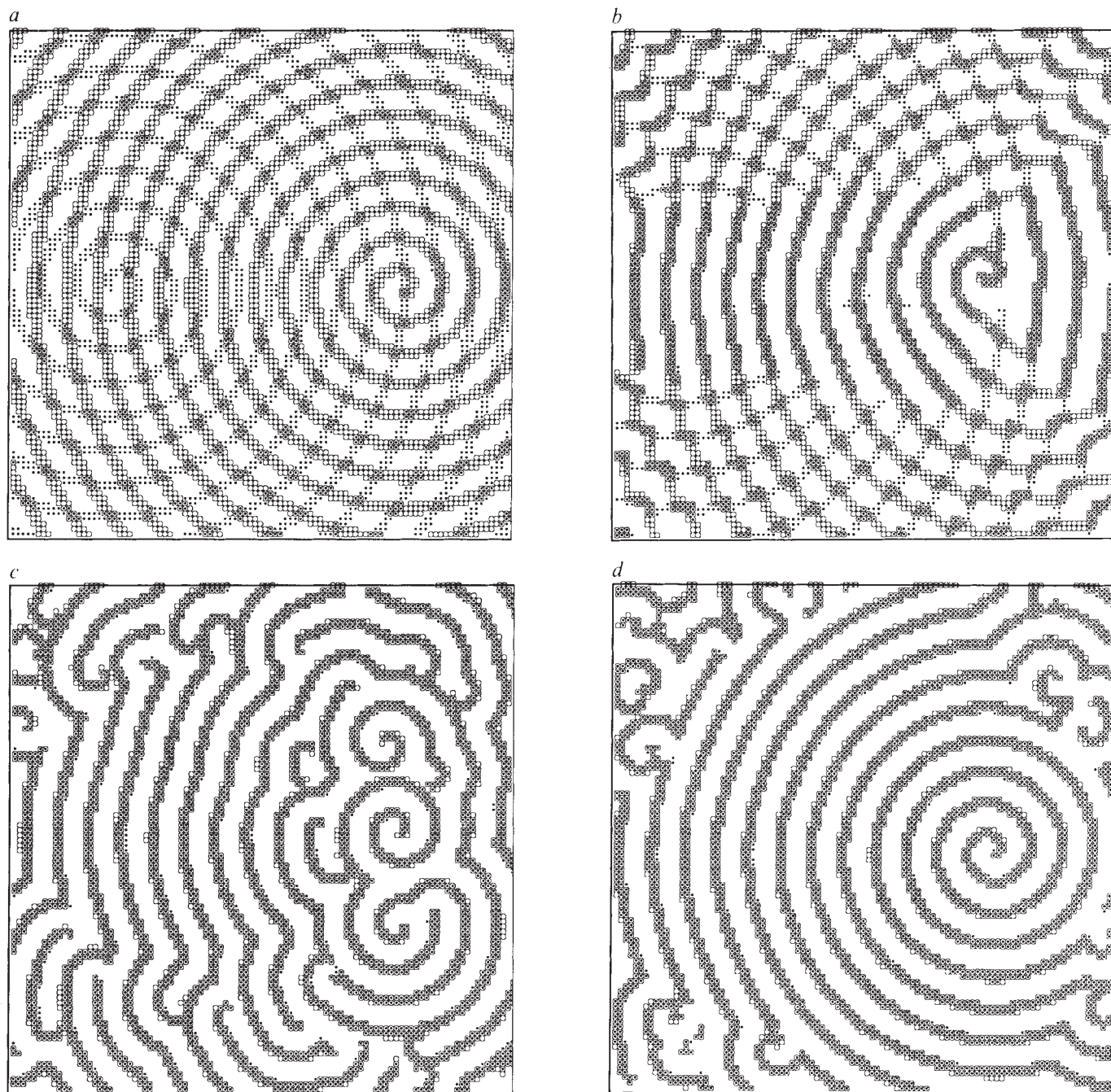


FIG. 4 Calculated evolution of spiral waves coupled across a model membrane. Open and filled circles show where oxidized catalyst is above 33% of its maximum concentration on each side of the membrane. The model for two coupled two-dimensional chemical-wave systems is based on a generic scheme for excitable media¹³: $\partial u/\partial t = f(u, v) + \nabla^2 u$, $\partial v/\partial t = g(u, v)$, where $f(u, v) = \varepsilon^{-1}u(1-u)(u-u_{th}(v))$, $g(u, v) = u-v$, and $u_{th} = (v+b)/a$. The two schemes are diffusively coupled by $\alpha(u_2 - u_1)$ and $\alpha(u_1 - u_2)$ terms for sides 1 and 2, respectively of the membrane, where α is transverse coupling

constant. Diffusion in the plane of and across the membrane occurs only for the fast variable u , corresponding to bromous acid; there is no diffusion of the slow variable v , corresponding to the fixed catalyst. *a*, Initially uncoupled spirals. *b*, Interacting waves after 2,000 iterations. *c*, Entrained waves after 16,000 iterations. *d*, With stronger coupling: regular entrained spiral after 16,000 iterations. The transverse coupling constant $\alpha = 0.35$ in *a-c* and 0.45 in *d*. Parameter values: $a = 0.55$, $\varepsilon = 0.02$ and $b = 0.010$ and 0.016 for higher- and lower-frequency spirals. Grid: 200×200 .

1:2 oscillations interspersed with 1:1 behaviour and, at a slightly different coupling strength, phase death⁴, where the wave patterns on one side of the membrane are completely extinguished by those on the other side.

Cross-membrane coupling of spatiotemporal patterns provides a new configuration for investigating communication between chemical systems. Transverse gradients of messenger species give rise to the spontaneous formation of spiral waves, which govern the subsequent evolution of entrainment (which includes the possibility of phase locking). Further studies of such cross-membrane coupling may provide insights into intercellular communication and coupling of excitable media in biological systems¹⁵. □

Received 22 February; accepted 25 March 1991.

1. Bar-Eli, K. *J. phys. Chem.* **88**, 3616–3622 (1984).
2. Györgyi, L. & Field, R. J. *J. phys. Chem.* **92**, 7079–7088 (1988).
3. Aronson, D. G., Doedel, E. J. & Othmer, H. G. *Physica D* **25**, 20–104 (1987).
4. Crowley, M. F. & Epstein, I. R. *J. chem. Phys.* **93**, 2496–2502 (1989).
5. Bar-Eli, K. *J. phys. Chem.* **94**, 2368–2374 (1990).
6. Zaikin, A. N. & Zhabotinsky, A. M. *Nature* **225**, 535–537 (1970).
7. Winfree, A. T. *Science* **181**, 937–939 (1973).
8. Field, R. J. & Noyes, R. M. *J. Am. chem. Soc.* **96**, 2001–2006 (1974).
9. Eisenberg, A. & Yeager, H. L. (eds) *Perfluorinated Ionomer Membranes*; Am. chem. Soc. Symp. Ser. **180** (American Chemical Society, Washington, DC, 1982).
10. Larter, R. *Chem. Rev.* **90**, 355–381 (1990).
11. Maselko, J. & Showalter, K. *Nature* **339**, 609–611 (1989).
12. Yatsimirskii, K. B., Tikhonova, L. P., Kovalenko, A. S. & Moshkovich, F. S. *J. phys. Chem.* **93**, 2848–2852 (1989).
13. Barkley, D., Kness, M. & Tuckerman, S. *Phys. Rev. A* **42**, 2489–2492 (1990).
14. Tyson, J. J. & Fife, P. C. *J. chem. Phys.* **73**, 2224–2237 (1980).
15. Winfree, A. T. *When Time Breaks Down* (Princeton Univ. Press, 1987).
16. Maselko, J. & Showalter, K. *J. phys. Chem.* **93**, 2774–2780 (1988).

ACKNOWLEDGEMENTS. We thank the US NSF and NATO (Scientific Affairs Division) for financial support of this work. K.S. thanks Z. Noszticzus for recommending that KBr be added to the reactant stream.

Molecular nitrogen emissions from denitrification during biomass burning

Thomas A. Kuhlbusch*, Jürgen M. Lobert†, Paul J. Crutzen† & Peter Warneck†

* Anorganisch-Chemisches Institut, Westfälische Wilhelms-Universität Münster, Wilhelm-Klemm-Strasse 8, 4400 Münster, Germany
† Departments of Biogeochemistry & Atmospheric Chemistry, Max-Planck-Institut für Chemie, PO Box 3060, 6500 Mainz, Germany

THE burning of biomass (forest vegetation, savannah grass, firewood and agricultural wastes) due to human activities in the tropics is an important source of nitrogen compounds in the atmosphere^{1–4}. A recent experimental study⁵ identified a gap of 35–60% in the nitrogen balance between its content in the fuel and that recovered in the ash and in gaseous emissions of NO_x, NH₃, HCN, CH₃CN and other nitriles, N₂O, higher-molecular-weight organic compounds and in the smoke. It was suggested that the missing compound had to be molecular nitrogen. We have now carried out appropriate experiments and find that molecular nitrogen is indeed the most important nitrogen species emitted from biomass burning, with the largest contribution coming from flaming combustion. The loss of nutrient nitrogen by biomass burning, which is ~10–50 Tg N yr⁻¹ or 5–50% of global nitrogen fixation, may be particularly important for tropical ecosystems.

An evacuable, cylindrical stainless steel chamber (volume, 13.6 dm³) with electropolished interior surfaces was used to burn different types of biomass in an artificial nitrogen-free atmosphere. Either helium-oxygen or argon-oxygen mixtures, 79:21% by volume, were used at ~1,200 hPa after evacuating and flushing the chamber several times. Side windows in the walls of the

container allowed observations of the experiments for which 0.5–1 g of various kinds of biomass were placed in a metal sample holder and ignited by resistance heating with a current of 180 A (4 V) for 5–20 s. Sample holders were made of stainless steel wire mesh or tantalum sheet. The former construction provided a better oxygen supply than the latter. The amounts of N₂ and CH₄ produced and of O₂ consumed (only in He-O₂ atmosphere) were determined by gas chromatography using a thermal conductivity detector. CO₂ and CO were measured by non-dispersive infrared gas analysis. The amounts of nitrogen and carbon that were volatilized were calculated from the differences between their concentrations in both biomass and ash, which were determined by standard ultimate analysis.

To ensure that N₂ emissions are not due to the release of trapped nitrogen from the cell cavities of the fuel and to demonstrate the tightness of the system, we stored several samples of biomass in the evacuated chamber for 20 h and refilled with nitrogen-free test gas. Without ignition there was no detectable evolution of N₂.

Table 1 summarizes the results of our experiments. Biomass materials studied included wood carvings (0.08), savannah grass *Trachypogon* (0.59), pine needles (0.88), hay (1.3), freshly cut and dried green grass (3.8) and clover (4.6), with mass percentage N-fractions shown in parentheses. The carbon contents ranged from 43 to 51% with an average of 46 ± 3%. Moisture contents were in the range 3–6%. Biomass combustion in argon-oxygen atmospheres generally produced bright flames, in contrast to helium-oxygen mixtures, which produced more smouldering combustion, presumably because the thermal conduc-

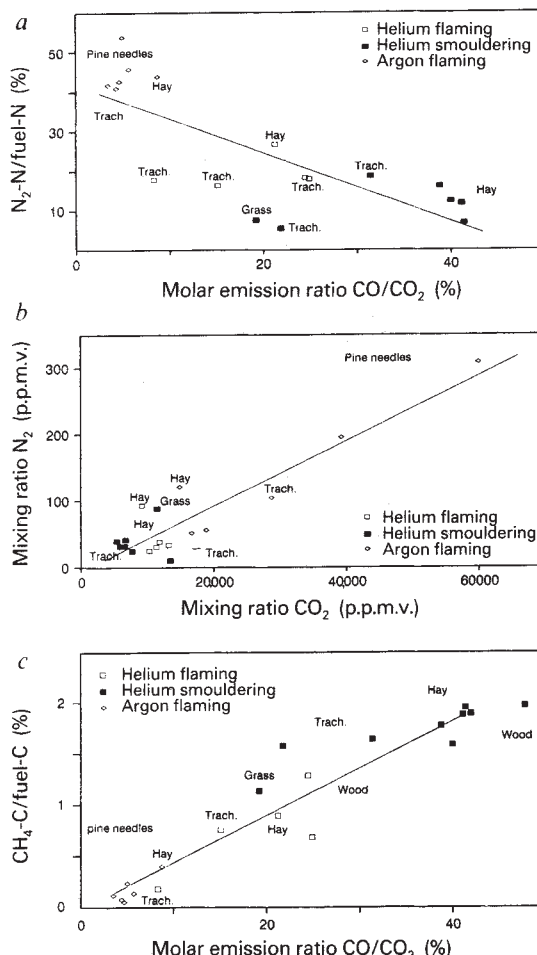


FIG. 1 Relations between N₂, CH₄, CO and CO₂ emissions observed in closed-chamber biomass burning experiments.

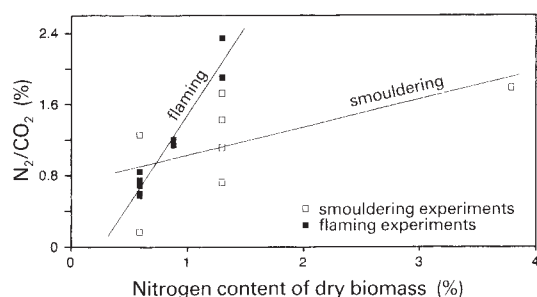


FIG. 2 N_2/CO_2 emission ratio in % (in terms of N/C) as a function of the biomass nitrogen content.

tivity and the associated heat losses are greater in helium. The thermal conductivity of argon is similar to that of nitrogen.

The combined emissions of CO , CO_2 and CH_4 do not fully account for the totally released carbon in most of the experiments. We ascribe this to the loss of material due to emissions of particulate matter and hydrocarbons not measured in this study which can, particularly in smouldering combustion, account for a large fraction of the products. The nitrogen balance is incomplete by necessity, as we do not report any nitrogen-containing compounds other than N_2 .

From all experiments we find that, on average, $31 \pm 20\%$ of the volatilized nitrogen was converted to N_2 . The N_2 yield of flaming combustion events was $46 \pm 15\%$ (total average). Compared with the fuel nitrogen, the N_2 yields were $23 \pm 16\%$ and $33 \pm 14\%$ (flaming combustions), respectively. Previously, we had shown that reduced compounds such as CH_4 are generated mainly during smouldering combustion, whereas the flaming stage results in higher oxidized products^{3,5}. The CO/CO_2 emission ratio therefore serves as a powerful indicator for the extent of smouldering combustion. In our experiments the CO/CO_2 ratios varied over a wide range of 0.03–0.48. Figure 1 combines various relationships between fuel-N, N_2 and CH_4 emissions on the one hand, and CO and CO_2 on the other hand. In Fig. 1a, the relative amounts of N_2 (in % of the fuel N-content) are plotted against the CO/CO_2 ratio; in Fig. 1b the mixing ratios of N_2 are plotted against those of CO_2 . Figure 1c shows

the production of methane (in % of the fuel C-content) and its dependence on the CO/CO_2 emission ratio. The negative slope of the linear regression line obtained in Fig. 1a indicates that flaming rather than smouldering combustion is the main source of N_2 . The plot in Fig. 1b shows a positive correlation and because CO_2 is formed primarily in the flame, this indicates again that N_2 is formed mostly during the flaming stage. Figure 1c shows a positive correlation between CH_4 and the CO/CO_2 ratio, which demonstrates that methane, unlike N_2 , is formed mainly during smouldering combustion; this agrees with previous results^{3,5}.

Figure 2 shows the relation between biomass nitrogen content and the N_2 emissions. There is a clear dependence on the fuel N content with a correlation coefficient of 0.98 for data derived from experiments with a prolonged flaming phase. In contrast, the results from smouldering combustions show only a slight positive correlation with considerable scatter.

CO/CO_2 ratios measured during field² and laboratory³ experiments are generally in the range of 5–15%. According to Fig. 1a, the corresponding yields of N_2 from fuel nitrogen are expected to vary between 40 and 30% with an average of $\sim 36\%$ corresponding to a CO/CO_2 ratio of 10%. This fraction, when added to that for other nitrogen-containing emissions (see the first paragraph), largely eliminates the gap in the nitrogen mass balance. Altogether, the measured and estimated emissions of the various forms of nitrogen, added to that remaining in the

TABLE 1 Carbon and nitrogen contents in biomass and ash and absolute emissions of CO , CO_2 , CH_4 and N_2

No.	Biomass	Comment	Elemental content				Emissions			
			Biomass (mg C)	Biomass (mg N)	Residue (mg C)	Residue (mg N)	CO (mg C)	CO ₂ (mg C)	CH ₄ (mg C)	N ₂ (mg N)
1	savannah grass	He sm	243.2	3.18	38.2	0.93	14.8	47.1	4.01	0.59
2	savannah grass	He fl	247.2	3.23	50.1	1.00	23.0	92.1	1.69	0.58
3	savannah grass	He fl	302.7	3.95	71.7	1.43	23.5	96.0	3.90	0.72
4	savannah grass	He sm	263.9	3.45	68.8	1.61	23.9	109.7	4.18	0.19
5	savannah grass	He fl	217.7	2.84	65.7	1.36	12.4	82.1	1.66	0.47
6	savannah grass	He fl	276.3	3.61	127.0	2.24	8.9	107.3	0.49	0.64
7	savannah grass	Ar fl	310.4	4.05	28.9	0.69	12.5	219.8	0.41	1.85
8	savannah grass	Ar fl	182.7	2.39	16.1	0.41	6.8	146.3	0.09	1.02
9	savannah grass	Ar fl	187.0	2.44	18.4	0.40	6.0	138.8	0.13	1.00
10	savannah grass	He sm	201.9	2.64	1.4	0.04	NM	NM	NM	0.72
11	hay	He sm	219.0	6.58	31.6	0.80	25.2	60.9	4.29	0.45
12	hay	He sm	219.0	6.57	43.9	1.54	22.0	53.5	4.14	0.77
13	hay	He sm	164.3	4.93	15.5	0.62	21.5	53.8	2.62	0.61
14	hay	He sm	154.0	4.62	28.2	0.80	16.6	42.7	2.74	0.74
15	hay	He fl	213.7	6.41	44.4	1.19	15.5	72.9	1.92	1.70
16	hay	Ar fl	165.6	4.97	13.0	0.76	10.0	114.7	0.66	2.18
17	pine needles	Ar fl	756.6	13.05	210.8	5.20	15.7	454.6	0.82	5.45
18	pine needles	Ar fl	374.8	6.47	34.3	1.31	15.0	301.0	0.87	3.48
19	grass	He sm	252.1	22.28	28.6	2.94	18.0	93.8	2.86	1.68
20	grass	He sm	419.6	37.08	99.9	9.04	NM	NM	NM	2.15
21	clover	He sm	423.9	43.92	130.1	12.71	NM	NM	NM	1.39
22	wood	He sm	450.6	0.73	58.9	0.18	50.5	105.6	8.90	<0.18
23	wood	He sm	289.3	0.47	0.0	0.00	38.6	91.8	5.49	<0.18
24	wood	He fl	227.1	0.37	13.2	0.03	25.3	86.5	2.91	<0.18

He, helium atmosphere; Ar, argon atmosphere; sm, smouldering predominant; fl, flaming predominant; NM, not measured.

residue, now account for almost 90% of the fuel nitrogen. The rest may be explained by the uncertainty of the measurements and the variability of individual combustion processes. A closer inspection of higher nitrogen-containing compounds in gaseous and particulate states might be helpful in improving the balance even further. The present results make clear, however, that N_2 represents the single most important nitrogen emission from biomass burning.

We now consider the impact of N_2 emissions from biomass burning on the global nitrogen balance of the tropical biosphere. There are two ways to extrapolate worldwide emissions. The easiest way is to consider the total prompt release of CO_2 to the atmosphere by biomass burning in the tropics, which was recently estimated as $1.8\text{--}4.7 \text{ Pg C yr}^{-1}$ (ref. 2). From our data in Fig. 1b, we obtain an average molar N_2/CO_2 emission ratio of 0.0047; the corresponding N/C mass ratio is 0.011. Combining these figures yields an estimated release of molecular nitrogen from global biomass burning of $20\text{--}52 \text{ Tg N yr}^{-1}$ with an average of 36 Tg N yr^{-1} .

Alternatively, the release of N_2 can be estimated from the global burning of plant nitrogen, which is quite uncertain, but was estimated by Crutzen and Andreae² to be in the range of $15\text{--}46 \text{ Tg N yr}^{-1}$. Multiplying by the average yield of 30–40% of N_2 derived in the present study, yields a N_2 release in the range of $5\text{--}20 \text{ Tg N yr}^{-1}$. To check for consistency we can also compare

the estimated N_2 emissions from biomass burning with emissions of $NO_y = NO + NO_2 + HNO_3$ from the same source. Our results suggest that ~36% of fuel nitrogen is emitted as N_2 compared with ~14% emitted as NO_y (ref. 5). Recent aircraft measurements⁶ of several trace gases over West Africa during the bushfire season have provided vertical profiles of NO_y and CO in the troposphere, from which we deduce a molar ratio $NO_y/CO \approx 0.04$. The CO emission rate due to tropical biomass burning is $120\text{--}510 \text{ Tg C yr}^{-1}$ (ref. 2). From the combination of these data we calculate a N_2 emission rate of $14\text{--}63 \text{ Tg N yr}^{-1}$ with an average of 38 Tg N yr^{-1} , consistent with our first estimate presented above.

The current estimate for the global rate of terrestrial, bacterial nitrogen fixation is $110\text{--}170 \text{ Tg N yr}^{-1}$ (ref. 7), and it is usually assumed that fixed nitrogen is lost entirely by microbial denitrification. The present results demonstrate that 12–47%, or alternatively 5–15%, of annually microbially fixed nitrogen is lost to the atmosphere in the form of N_2 due to 'pyrodenitrification'. The percentage is higher in tropical regions where ~90% of the global biomass burning takes place. Thus, the emissions of molecular nitrogen from biomass burning in these regions can cause important losses of fixed nitrogen to the ecosystems, especially from savannah and agricultural soils, reducing the availability of an essential nutrient for plants. □

Received 14 January; accepted 25 March 1991.

- Crutzen, P. J., Heidt, L. E., Krasnec, J. P., Pollock, W. H. & Seiler, W. *Nature* **282**, 253–256 (1979).
- Crutzen, P. J. & Andreae, M. O. *Science* **250**, 1669–1678 (1990).
- Lobert, J. M. *et al.* in *Global Biomass Burning* (ed. Levine, J.S.) (MIT Press, Cambridge, in the press).

- Warneck, P. *Chemistry of the Natural Atmosphere* (Academic, San Diego 1988).
- Lobert, J. M., Scharffe, D. H., Hao, W. M. & Crutzen, P. J. *Nature* **346**, 552–554 (1990).
- Marenco, A., Medale, J. C. & Prieur, S. *Atmos. Environ.* **24A**, 2823–2834 (1990).
- Rossall, T. in *Some Perspectives of the Major Biogeochemical Cycles SCOPE 17* (ed. Likens, G. E.) 25–49 (Wiley, Chichester, 1981).

A possible normal-fault rupture for the 464 BC Sparta earthquake

R. Armijo*, H. Lyon-Caen* & D. Papanastassiou†

* Institut de Physique du Globe, 4 place Jussieu, 75252 Paris Cedex 05, France

† National Observatory of Athens, PO Box 20048, 11810 Athens, Greece

SURFACE ruptures have been identified for some normal-faulting earthquakes in the Aegean region^{1–3}, but for most historical earthquakes the associated faults are unknown. This hampers the evaluation of the rates and styles of present-day deformation, and the assessment of seismic hazard in the region⁴. Here we examine the famous earthquake that destroyed Sparta in 464 BC. Using SPOT satellite images and fieldwork, we have mapped a 20-km-long normal fault scarp trending approximately north–south, a few kilometres east of the ancient city. Our observations, combined with an examination of historical descriptions of the earthquake damage, suggest that the Sparta earthquake ruptured this fault scarp in an event of magnitude $M_s \approx 7.2$. The Holocene slip rate and the recurrence time for such large events on the Sparta fault would be $\sim 1 \text{ mm yr}^{-1}$ and $\sim 3,000 \text{ yr}$, respectively.

To many historians of the Greek classical period^{5–8}, the earthquake that destroyed Sparta in ~464 BC was an important reference. Ducat⁹ points out that there exist three independent contemporaneous descriptions of the 464 BC earthquake: by Thucydides, who reports the event in his History of the Peloponnesian War⁵, and whose description has been followed by that of Pausanias⁶; by Ephorus, who apparently provided the source for Diodorus of Sicily⁷; and an unidentified hellenistic author, followed later by Cicero, Pliny and Plutarch⁸. The earthquake

was large, according to Thucydides, who refers to it as the "great earthquake in Sparta" (ref. 5, I, 128). Diodorus gives an estimate of the death toll and an indication of multiple shocks: "the houses collapsed from their foundations and more than twenty thousand Lacedemonians perished. And since the tumbling down of the city and the falling in of the houses continued uninterruptedly over a long period, many persons were caught and crushed in the collapse of the walls" (ref. 7, XI, 63). Plutarch provides an idea of the local effects and details the devastation:

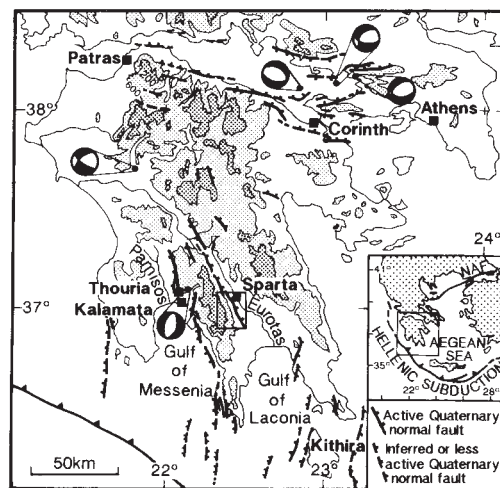
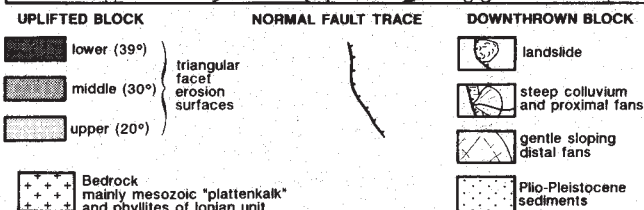
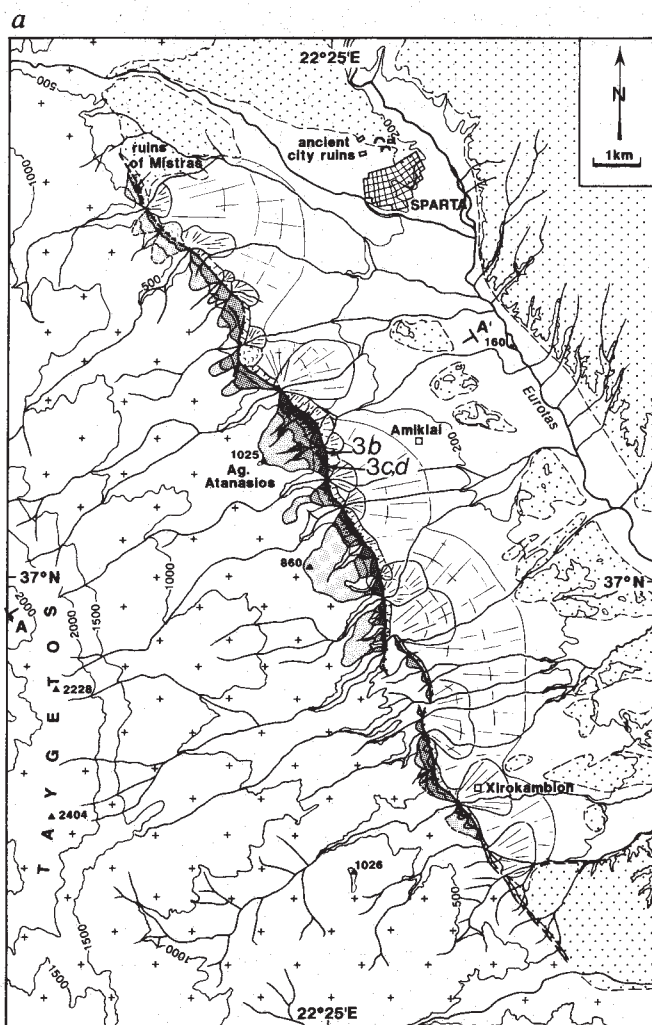


FIG. 1 Seismotectonic map of Peloponnesus (modified from ref. 1). Focal mechanisms from refs 1, 3. Insert shows the location of Fig. 2a; NAF denotes the North Anatolian fault.



"a greater earthquake than any before reported rent the land of the Lacedaemonians into many chasms, shook Taygetus so that sundry peaks were torn away, and demolished the entire city with the exception of five houses" (ref. 8, XVI, 4). Plutarch also reports the existence, five centuries later, of "archaeological evidence" of the events: "Their tomb, even down to the present day, they call Seismatias" (ref. 8, XVI, 5). Clearly, the Sparta earthquake was large and destructive, probably accompanied by ground fissures, landslides, rock falls and other surface effects. This is compatible with a maximum intensity $I \geq X$ MSK (ref. 10) on the Mercalli scale. The death toll was probably very high, but the figure given by Diodorus (20,000 or about half of the inhabitants of the city¹¹) seems to be overestimated^{9,12}.

The earthquake had immediate political consequences. Thucydides reports "the simultaneous revolt and secession to Ithome of the Helots and of some of the Perioeci (those of Thouria and of Aithaia)" (ref. 5, I, 101). According to Diodorus, "The Helots and Messenians... stood in fear of the eminent position and power of Sparta; but when they observed that the larger part of them had perished of the earthquake, they held in contempt the survivors, who were few" (ref. 7, XI, 63.4). Although the importance of the revolt of the Helots of Laconia (southeast Peloponnese) is not well established, all scholars agree on the significance of the revolt and secession of the Messenians (Helots and Perioikoi of Messenia, southwest Peloponnese) after the earthquake: the so-called Third Messenian War¹². The historical record thus strongly suggests that the destruction caused by the earthquake was much less in the Pamisos valley, where the Messenians were settled, than in Sparta and the Eurotas valley (Fig. 1). Given the short distance between the two valleys (Thouria is only 40 km west of Sparta), we infer that the 464 BC earthquake was not of subduction type but a shallow crustal event located not far from Sparta. A comparable example of local destruction is the normal-faulting earthquake that heavily damaged Kalamata in 1986¹.

The eastern front of the Taygetos range is bounded by an east-dipping, 60-km-long, normal-fault system made up of several left-stepping, NNW-SSE striking, en échelon segments (Fig. 1). This fault system is similar, but antithetic, to the west-dipping system formed by the Kalamata fault and the en échelon normal-fault segments located offshore of the Mani peninsula¹ (Fig. 1). The rock types that crop out on the Taygetos range near Sparta are mainly phyllites of Permian to early Triassic age and late Senonian-Eocene limestones of the Ionian unit¹³. These latter limestones mark remarkably well the normal-fault landforms (Fig. 2a), which are very similar to features seen more clearly in other extensional environments such as the Basin and Range or Tibet¹⁴⁻¹⁶. East and south of Sparta, a 20-km-long fault segment with particularly young morphology¹⁷ (here referred to as the Sparta fault) is composed of three sub-segments (Fig. 2a). The mountain front has a maximum local relief of more than 700 m at the central fault sub-segment (Fig. 2b). The front is formed by steep triangular/trapezoidal facets separated by distinct V-shaped valleys and 'wineglass canyons' (Fig. 3a). Three sets of triangular facets can be discerned, with maximum heights of about 725, 450 and 250 m, and slopes of ~20°, 30° and 40°, respectively (Figs 2, 3a). The decrease in facet heights towards the north and south defines the length and the individuality of the Sparta fault segment. The fault trace is linear, continuous and follows the mountain-piedmont junction (Fig. 2a). The large alluvial fans on the piedmont are mostly undissected and still active, but there is some young fanhead entrenchment, ~2-4 m deep near the fault trace. All of these observations suggest recent, sustained uplift of the front, and young faulting activity¹⁸. Furthermore, the existence along the three sub-segments of the Sparta fault of an almost continuous fresh scarp cutting across bedrock and indurated conglomerates (Fig. 3b, c, d) is definite evidence for recent, most probably seismic, faulting events. Although hanging-wall erosion may have increased the scarp height at some places, the uniform

FIG. 2 a, Map of Sparta fault. Topography from ref. 21. Arrows are locations of Fig. 3b and c. b, Interpretative section at A-A'. Geology simplified from ref. 13. Total throw is probably greater than 2 km. Dashed fault is inactive.

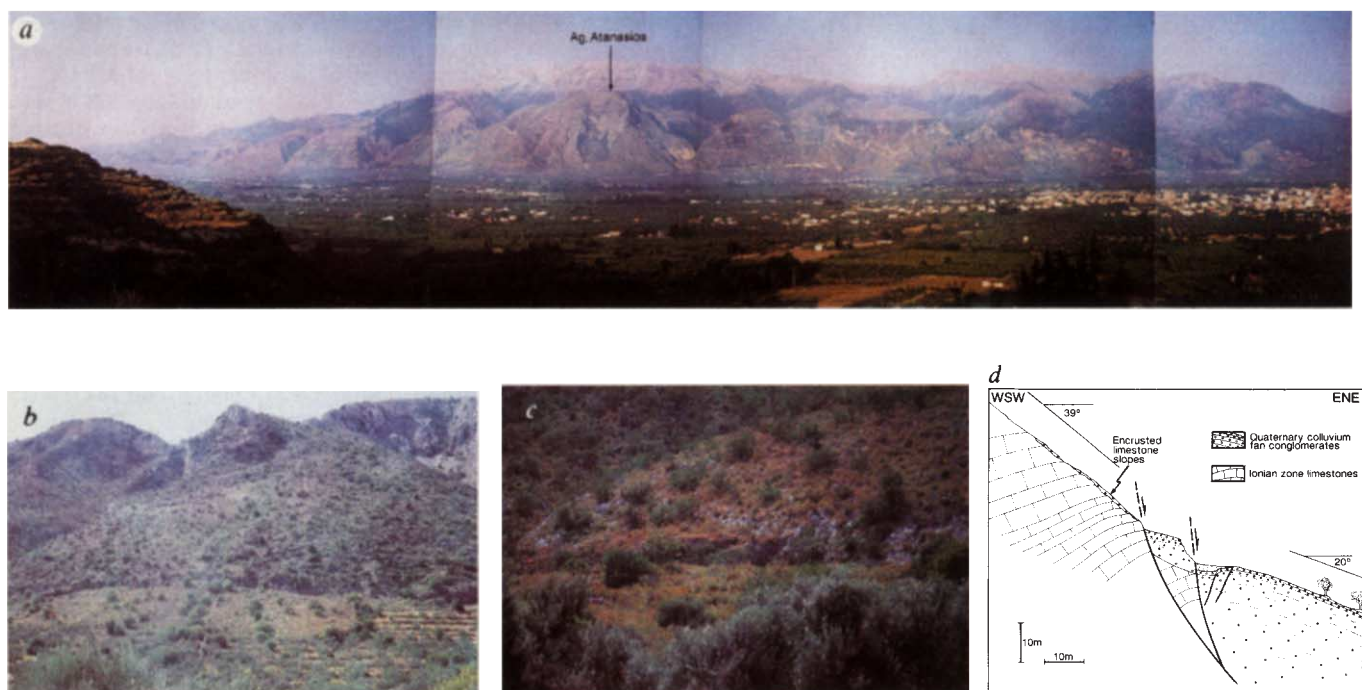


FIG. 3 a, Sparta mountain front. Panoramic view from the west. b, Fault scarp along base of triangular facet. c, Detail of scarp with uplifted

conglomerates. d, Section of Sparta fault scarp at c.

shape of the scarp along strike implies a reliable maximum throw (vertical displacement) of ~ 10 – 12 m on the central fault sub-segment. Not having dug trenches, we cannot assess unambiguously whether this maximum throw corresponds to one or to many events. The observation, however, that a conglomerate wedge lies against the bedrock along a smaller inner scarp ($\sim 1/3$ of the total throw), and that this wedge overhangs a main, fresher outer scarp ($\sim 2/3$ of the total throw) (Fig. 3c, d), suggest that the total throw resulted from cumulation of at least two or three faulting events. In view of the historical record and of this field evidence, we suggest that the 464 BC earthquake could correspond to the last of these events.

To characterize a possible seismic rupture along the Sparta fault, we assume the fault geometry at depth to be the same as that of the Kalamata fault (Fig. 1), whose dip of $\sim 45^\circ$ is well constrained down to a depth of 10 km (ref. 1). Such a depth of the brittle/ductile transition zone seems to be implied by most studies of recent earthquakes in the region¹⁹. A rupture of the Sparta fault (20×14 km) with an average slip of 10 m (total displacement corresponding to the observed scarp) and shear modulus $\mu = 3.23 \times 10^{10}$ N m⁻² would yield an earthquake with

moment $M_0 = 9 \times 10^{19}$ N m. The large slip compared to the width of the fault would require a particularly high static stress drop (~ 17 MPa). An event with the same fault surface but a third of the displacement above yields a moment $M_0 = 3 \times 10^{19}$ N m, similar to the 1980 earthquake at Irpinia, Italy²⁰. Our observations suggest that such an event is the most likely maximum-magnitude event along the Sparta fault. On the other hand, an average slip of only 0.5 m on the Sparta fault would yield a moment similar to those of the largest recent earthquakes of Thessaloniki (1978) and Corinth (1981) ($M_0 = 0.52$ and 0.68×10^{19} N m)^{2,3}. We conclude that the 464 BC earthquake most probably had a moment M_0 of the order of 3.0×10^{19} N m and a magnitude $M_s = 7.2$. A comparison of the scarp along the Sparta fault with other scarps with similar morphology in southern Peloponnese and Crete (R.A., H.L.-C. and D. Papanastassiou, manuscript in preparation) suggests that these scarps are of Holocene age (≥ 10 kyr). In this event the slip rate of the Sparta fault would be ~ 1 mm yr⁻¹, and, if at least three events produced the observed 10-m-high scarp ($M_s \approx 7.2$ each), the corresponding maximum recurrence time for large earthquakes would be of the order of 3,000 yr. $\square$

Received 13 December 1990; accepted 18 March 1991.

1. Lyon-Caen, H. *et al.* *J. geophys. Res.* **93**, 14967–15000 (1988).
2. Soufleris, C., Jackson, J. A., King, G. C. P., Spencer, C. P. & Scholz, C. H. *Geophys. J. R. astr. Soc.* **68**, 429–458 (1982).
3. Jackson, J. A. *et al.* *Earth planet. Sci. Lett.* **57**, 377–397 (1982).
4. Jackson, J. A. & McKenzie, D. P. *Geophys. J.* **93**, 45–73 (1988).
5. Thucydides *History of the Peloponnesian War* (Penguin, Melbourne, 1960).
6. Pausanias *Description of Greece* (Macmillan, London, 1898).
7. Diodorus of Sicily *Volume IV* (Harvard University Press, Cambridge, 1961).
8. Plutarch *Lives: Cimon* (Harvard University Press, Cambridge, 1968).
9. Ducat, J. *Actes IV Recontres Internationales d'Archéologie et d'Histoire*, Antibes, 73–85 (Centre National de la Recherche Scientifique, Paris, 1983).
10. Galanopoulos, A. G. *Greece, A Catalog for Shocks with $I_0 \geq VII$ for the years before 1800* (ed. Athens University) (Athens, 1961).
11. Toynbee, A. *Some Problems of Greek History* (Oxford University Press, 1969).

12. Cartledge, P. *Sparta and Lakonia, A Regional History 1300–1362 BC* (Routledge and Kegan Paul, London, 1979).
13. Geological map of Greece, scale 1:50,000 (Institute of Geology and Mineral Exploration, Athens, 1983).
14. Wallace, R. E. *J. Res. US Geol. Surv.* **6**, 637–650 (1978).
15. Hamblin, W. K. *Geology* **4**, 619–622 (1976).
16. Armijo, R., Tapponnier, P., Mercier, J. L. & Han, T. L. *J. geophys. Res.* **91**, 13,803–13,872 (1986).
17. Dufaure, J. *J. Rev. Géol. Phys.-Géol. Dyn.* **19**, 27–58 (1977).
18. Bull, W. B. *US geol. Surv. Open-File Rep.* **87-673**, 192–292 (1987).
19. Jackson, J. A. & White, N. J. *J. struct. Geol.* **11**, 15–36 (1989).
20. Deschamps, A. & King, G. C. P. *Earth planet. Sci. Lett.* **62**, 296–304 (1983).
21. Hellenic Army Geographical Service Topographic map 1:50,000 (1977).

ACKNOWLEDGEMENTS. We thank P. Tapponnier for critically reading the manuscript and J. Jackson for a constructive review. G. Aveline drafted the figures. This work was funded by Institut National des Sciences de l'Univers (INSU-CNRS).

Aseismicity in the lower mantle by superplasticity of the descending slab

Eiji Ito & Hiroki Sato

Institute for Study of the Earth's Interior, Okayama University, Misasa, Tottori-ken 682-01, Japan

It has been suggested¹⁻⁷ that deep earthquakes in subduction zones may be caused by mineralogical transformations in the subducting slab. These transformations, from olivine to modified spinel to spinel, are also thought to affect the rheology of the descending slab²⁻⁴. Experiments in the system $\text{Mg}_2\text{SiO}_4\text{--Fe}_2\text{SiO}_4$ (ref. 8) have shown that at still higher pressure, coinciding with the 670-km seismic discontinuity between the upper and lower mantle, spinel dissociates to a fine-grained mixture of perovskite plus magnesiowüstite. Here we argue that the dissociation product in the mantle will also be very fine-grained, and that its eutectoid texture, combined with the low temperature in the slab, will prevent grain growth. We note (as have others^{9,3}) that fine-grained materials at moderately high temperatures and low strain rates exhibit superplastic behaviour; the resulting viscous behaviour of the slab below the spinel dissociation boundary may therefore account for the observed absence of seismicity below ~700 km depth, even if slabs do penetrate into the lower mantle.

Recent tomographic inversions of seismic data have indicated high-velocity anomalies that may correspond to a subducted slab penetrating into the lower mantle^{10,11}. One objection to this idea of slab penetration is the widely observed lack of seismic activity below ~700 km depth. O'Connell⁹ suggested that, among other possibilities, the aseismicity could be due to the low effective viscosity of the fine-grained mixture of oxides that he assumed would form by spinel dissociation in the lower mantle. Rubie³ also pointed out that aseismicity of the slab in the upper mantle could arise from superplasticity associated with the fine-grained products of the transformations from olivine to modified spinel to spinel. Here we re-visit the idea of rheology-induced aseismicity, but with the benefit of new experimental constraints⁸ on the nature of the post-spinel transformation.

The experiments of ref. 8 showed that mantle spinel ($\text{Fe}/(\text{Mg} + \text{Fe}) = 0.1\text{--}0.15$) dissociates at ~23 GPa and 1,600 °C into an assemblage of perovskite (Pv) and magnesiowüstite

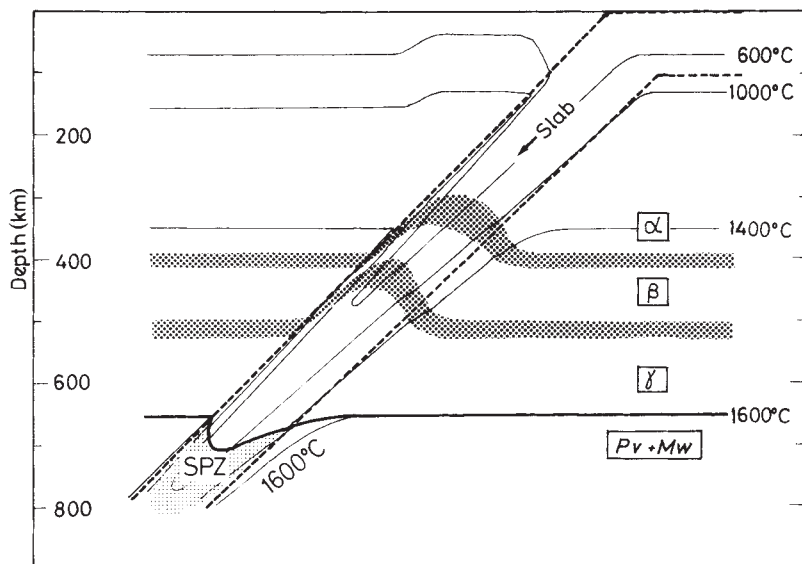
(Mw) within a very narrow pressure interval (less than 0.14 GPa). Sharp changes in seismic velocity and density observed at the 670-km discontinuity are reasonably attributed to this dissociation in the peridotitic mantle. The phase boundary has a negative Clapeyron slope of approximately $-3 \text{ MPa } ^\circ\text{C}^{-1}$ (ref. 8). Therefore, the physical boundary of dissociation in the relatively cold slab should be convex towards greater depths (Fig. 1). This intrusion of low-density spinel into the lower mantle can produce a large buoyant force on the slab.

The product of spinel dissociation (Pv + Mw) is a fine-grained composite with a eutectoid texture (Fig. 2). In the course of dissociation, nucleation and growth of Mw (Pv) produces a zone of $(\text{Mg}, \text{Fe})\text{SiO}_3$ ($(\text{Mg}, \text{Fe})\text{O}$) around the nucleation site. Around this zone in turn, nucleation and growth of Pv (Mw) creates a zone of $(\text{Mg}, \text{Fe})\text{O}$ ($(\text{Mg}, \text{Fe})\text{SiO}_3$). Thus the two phases should be distributed alternately in the product. We observe that the assemblage formed after 1 h at 1,600 °C and 24 GPa is an aggregate of grains 0.1–2.0 μm in size (Fig. 2). The corresponding product in the slab may contain smaller grains than this because of the lower temperature of the transformation (Fig. 1).

The deformation of such fine-grained materials at moderately high temperatures ($T/T_m > 0.5$, where T_m is the absolute solidus) and low strain rates results in superplastic behaviour^{12,13}, in which grain-boundary sliding is the dominant deformation mechanism. From experiments on fine-grained limestone, Schmit *et al.*¹³ specified the regime of superplastic deformation. They showed that the flow stress at any given strain rate is sensitive to grain size in the superplastic regime, and that the expected flow stress for grain sizes less than 10 μm is extremely low; at $T = 0.54 T_m$ and a strain rate of 10^{-10} s^{-1} , for example, the flow stress is 0.3 MPa. For a lower strain rate and a smaller grain size, the flow stress becomes lower still. Superplasticity in fine-grained ceramic oxides has been well established^{14,15}.

The assemblage of Pv and Mw produced in the slab satisfies the conditions for superplasticity: grain sizes of less than several micrometres, relatively high temperatures ($T/T_m > 0.5$; for Fig. 1, $T_m \approx 2,500 \text{ K}$ (ref. 16)) and low strain rates ($\sim 10^{-14} \text{ s}^{-1}$ in the mantle). Grain coarsening of the assemblage would occur during further subduction. It should be emphasized, however, that at these strain rates, even grain sizes of ~100 μm may still produce superplastic behaviour^{13,17}. Because Pv grains are interconnected (unlike the essentially isolated Mw grains; Fig. 2), grain-boundary migration of Pv will be the dominant mechanism of grain coarsening. We can make a rough estimate of the grain

FIG. 1 Phase transformations in a descending slab. The upper mantle is composed of α (olivine), β (modified spinel) and γ (spinel) phases. The spinel dissociation into perovskite (Pv) and magnesiowüstite (Mw) takes place within a very narrow depth interval (bold line), whereas the α – β and β – γ transformations occur within the hatched areas. The mantle geotherm is from Sato *et al.*²⁶ and Ito and Katsura²⁷. The temperature of the slab is calculated according to Schubert *et al.*²⁰. Because of the positive Clapeyron slopes of the α – β and β – γ transformations, the transitional regions become shallower in the cold slab. (The possibility of a zone of metastable olivine in the slab^{6,28} is neglected for simplicity.) The negative Clapeyron slope of the spinel dissociation at ~650 km depth causes the boundary to be at greater depths within the slab. Beneath this boundary, the fine-grained assemblage of Pv + Mw (region SPZ) satisfies the conditions for superplasticity. Therefore, earthquakes may occur only up to ~700 km depth in the slab. This is consistent with the maximum depth observed for earthquakes.



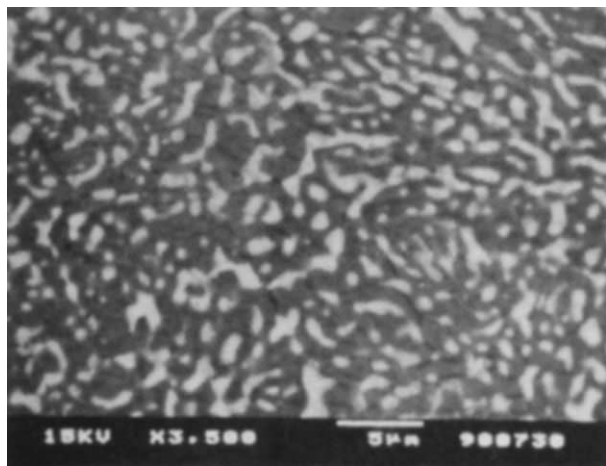


FIG. 2 Back-scattered electron image of the assemblage of perovskite (dark) and magnesiowüstite (light), showing a eutectoid (mutual intergrowth) texture. The sample was formed from a coarse-grained (10–20 µm) spinel (Fe/(Mg + Fe)=0.1) heated to 1,600 °C at 24 GPa for 1 h. The grain size of the composite is mostly less than 3 µm.

growth rate of Pv by the kinetic law¹⁸ $d^3 \approx kt$, where d is the grain size at time t , and k , a growth-rate constant, is given by $k = k_0 \exp(-Q/RT)$ (k_0 is a pre-exponential factor, Q is an activation energy and R is the gas constant). Adopting $d \approx 1 \mu\text{m}$ at $t = 1 \text{ h}$ (see Fig. 2), the time taken for grains to grow to $\sim 100 \mu\text{m}$ is $\sim 10^2 \text{ yr}$ at 1,600 °C. As Q is several hundreds of kJ mol^{-1} for oxides (for example, $Q = 253 \text{ kJ mol}^{-1}$ for ZnO (ref. 18) and 580 kJ mol^{-1} for ZrO_2 (ref. 19)), the growth time in a slab could be several orders of magnitude larger than this because the temperatures are several hundreds of degrees lower than 1,600 °C (Fig. 1)²⁰. The existence of a second phase (Mw) also suppresses grain growth substantially by a pinning effect^{14,21}. Although superplastic flow in the slab can itself enhance grain growth^{19,22}, this effect should be negligible because of the small strain rate (10^{-14} s^{-1})¹⁴. Therefore an extensive superplastic zone should be formed in the slab beneath the spinel dissociation boundary, especially in the cooler region.

In this zone, the actual yielding stress is probably less than 1 MPa, so that virtually no elastic energy can be stored^{13,17}. This may be the main reason why no earthquakes are observed in the lower mantle below $\sim 700 \text{ km}$ depth. The sudden disappearance of seismic activity at this depth is also consistent with the sharpness of the spinel dissociation boundary (width of less than 5 km)^{8,23}. There is much debate about whether slabs penetrate into the lower mantle²⁴; our work suggests that aseismicity below $\sim 700 \text{ km}$ is not necessarily evidence against slab penetration, as also suggested by O'Connell⁹.

In the lower mantle there would exist a density contrast between the cold slab and the hot surrounding mantle. Ductility in the superplastic zone would, however, prevent the higher density of the slab from providing a driving force for subduction. In other words, the spinel dissociation determines the lower bound of the rigid body of the slab. The force acting on the slab then consists of a large negative buoyancy at depths above 400 km, owing to the density contrast from a large temperature difference ($\geq 500 \text{ °C}$ in some regions; Fig. 1) and to the transformation from olivine to modified spinel, and a fairly large positive buoyancy owing to penetration of the spinel zone into the lower mantle. The magnitude of these forces depends on the age, the dip angle and the descent speed of the slab. For the simple model shown in Fig. 1, the intermediate region of the slab between depths of 400 and 670 km should experience strong compression. This might account for the down-dip compression

sional earthquakes observed in seismic studies^{5,25}. Negative buoyancy from a possible metastable region of olivine⁶ will not change this conclusion. □

Received 11 September 1990; accepted 22 March 1991.

1. Kirby, S. H. *J. geophys. Res.* **92**, 13789–13800 (1987).
2. Green, H. W., Young, T. E., Walker, D. & Scholz, C. H. *Nature* **348**, 720–722 (1990).
3. Rubie, D. C. *Nature* **308**, 505–508 (1984).
4. Rubie, D. C. & Brearley, A. J. *Nature* **348**, 628–631 (1990).
5. Frohlich, C. A. *Rev. Earth planet. Sci.* **17**, 227–254 (1989).
6. Sung, C.-M. & Burns, R. G. *Tectonophysics* **31**, 1–32 (1976).
7. Liu, L.-G. *Phys. Earth planet. Inter.* **32**, 226–240 (1983).
8. Ito, E. & Takahashi, E. *J. geophys. Res.* **94**, 10637–10646 (1989).
9. O'Connell, R. J. *Tectonophysics* **38**, 119–136 (1977).
10. Fischer, K. M., Jordan, T. H. & Creager, K. C. *J. geophys. Res.* **93**, 4773–4783 (1988).
11. Kamiya, S., Miyatake, T. & Hirahara, K. *Geophys. Res. Lett.* **15**, 828–831 (1988).
12. Boullier, A. M. & Gueguen, Y. *Contr. Miner. Petrol.* **50**, 93–104 (1975).
13. Schmid, S. M., Boland, J. N. & Paterson, M. S. *Tectonophysics* **43**, 257–291 (1977).
14. Chen, I.-W. & Xue, L. A. *J. Am. Ceram. Soc.* **73**, 2585–2609 (1990).
15. Kim, W.-J., Wolfenstine, J. & Sherby, O. D. *Acta metall. mater.* **39**, 199–208 (1991).
16. Ito, E. & Takahashi, E. *Nature* **328**, 514–517 (1987).
17. Heuer, A. H., Tighe, N. J. & Cannon, R. M. *J. Am. Ceram. Soc.* **63**, 53–58 (1980).
18. Gupta, T. K. & Coble, R. L. *J. Am. Ceram. Soc.* **51**, 521–525 (1968).
19. Nieh, T.-G. & Wadsworth, J. *J. Am. Ceram. Soc.* **72**, 1469–1472 (1989).
20. Schubert, G., Yuen, D. A. & Turcotte, D. L. *Geophys. J. R. astr. Soc.* **42**, 705–735 (1975).
21. Harkulich, T. M., Magder, J., Vukasovich, M. S. & Lockhart, R. J. *J. Am. Ceram. Soc.* **49**, 295–299 (1966).
22. Wakai, F. & Kato, H. *Adv. Ceram. Mat.* **3**, 71–76 (1988).
23. Richards, M. A. & Wicks, C. W., Jr. *Geophys. J.* **101**, 1–35 (1990).
24. Frohlich, C. & Grand, S. P. *Nature* **347**, 333–334 (1990).
25. Zhou, H.-W. & Clayton, R. W. *J. geophys. Res.* **95**, 6829–6851 (1990).
26. Sato, H., Sacks, I. S., Takahashi, E. & Scarfe, C. M. *Nature* **336**, 154–156 (1988).
27. Ito, E. & Katsura, T. *Geophys. Res. Lett.* **16**, 425–428 (1989).
28. Iidaka, T. & Suetsugu, D. *Eos* **71**, 1574–1575 (1990).

ACKNOWLEDGEMENTS. We thank T. Katsura for the micrograph. We are grateful to O. Ohtaka, S. Akimoto and Y. Matsui for their support. We are indebted to F. Wakai and T. Suzuki for informative discussion. This work was supported by the Ministry of Education, Science and Culture, Japan.

Venom-conducting teeth in a Triassic reptile

Hans-Dieter Sues

Department of Paleobiology, National Museum of Natural History, Washington, DC 20560, USA

I REPORT here the discovery of highly distinctive reptilian teeth of early Late Triassic age from the Newark Supergroup of Virginia¹, which are distinguished by the development of a deeply infolded median groove on both the labial and lingual surfaces of the blade-like crowns. On the basis of the close structural similarity to comparable features in the two living species of the lizard *Heloderma* and on the poison-fangs in extant venomous snakes^{2–5}, it is suggested that these grooves functioned in venom conduction. This would represent the earliest instance of the use of oral toxins among reptiles (comprising diapsids and turtles) recorded to date.

The new fossils form part of a recently discovered assemblage of early Late Triassic vertebrates from the Richmond basin of the Newark Supergroup of Virginia (United States)¹. The mostly disassociated but excellently preserved bones and teeth occur in a massive calcareous mudstone together with root traces, coalified plant debris, and numerous small calcareous nodules. The vertebrate assemblage is quite varied and includes several previously unknown taxa. The most common faunal element is the eucynodont synapsid *Boreogomphodon*, which is most closely related to certain genera from the Triassic of Brazil and Zambia.

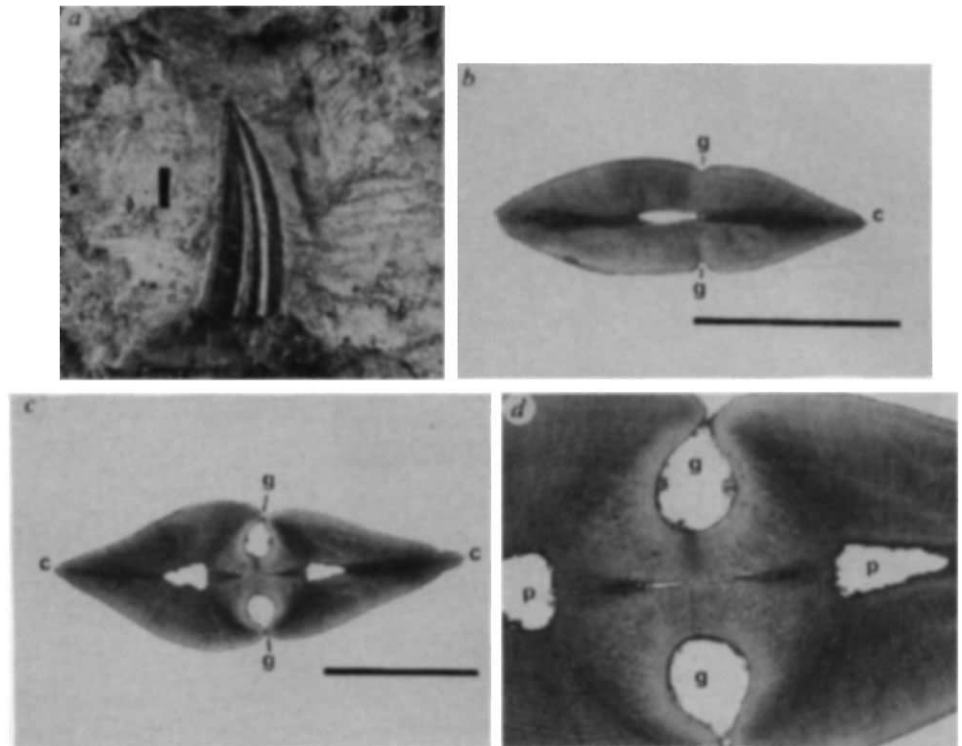
Uatchitodon gen. nov.

Type-species. *Uatchitodon kroehleri* nov.

Etymology. *Uatchit*, the ancient cobra goddess of Lower Egypt, and ὀδών (Greek; Ionic variant ὀδών), tooth.

Diagnosis. Tooth crowns strongly compressed labiolingually, recurved and typically with serrated anterior and posterior cutting edges. Both labial and lingual surfaces with deeply infolded median grooves.

FIG. 1 *Uatchitodon kroehleri*, new species. *a*, USNM 448611 (holotype), isolated tooth crown as preserved in matrix. *b*, USNM 448612, transverse section near the tip of tooth crown; note shallow grooves (*g*), cutting edge (*c*) and undivided pulp cavity. *c*, USNM 448619, transverse section at mid-height of another tooth crown with deeply infolded grooves (*g*) on the labial and lingual surfaces and distinct cutting edges (*c*). *d*, Magnified detail of *c*; note modification of pulp cavity (*p*). Scale bars, 1 mm.



Uatchitodon kroehleri sp. nov.

Etymology. For P. A. Kroehler, who found the holotype and several other specimens.

Holotype. USNM (National Museum of Natural History) 448611, isolated tooth (Fig. 1*a*).

Referred material. USNM 448603–448610, 448621–448622 and 448624, teeth; USNM 448612–448619, thin-sections of tooth fragments.

Locality. USNM locality 39981 (1988–1), Chesterfield County, Virginia.

Horizon. Tomahawk Creek Member, Turkey Branch Formation, Newark Supergroup (Late Triassic: early middle Carnian)¹.

Diagnosis. Type and only known species of the genus as diagnosed above.

Only isolated teeth (mostly crowns) have been found to date but their singular structure sets them apart from those of any other known tetrapod. They reach an average crown height of about 10 mm but several fragments indicate the existence of larger teeth. The thecodont mode of tooth implantation indicated by the root of USNM 448624 suggests reference to the Amniota. Although the precise phylogenetic position of *Uatchitodon* remains to be determined, its teeth most closely resemble those of carnivorous archosauriform reptiles in overall form and structure and are unlike those of any Late Triassic synapsid. The pointed crowns are recurved and strongly flattened labiolingually (Fig. 1*a*). The posterior and the more apical portion of the anterior cutting edge are distinctly serrated on all but two teeth found to date, usually with six or seven denticles per millimetre. The thin enamel of the crowns is smooth except for some wavy banding on several teeth. Polished transverse sections of tooth crowns show numerous concentric growth lines in the dentine, which probably reflect periods of quiescence during the deposition of this tissue².

The diagnostic feature of the teeth is the presence of a deeply infolded median groove along both the labial and lingual surfaces of the crown. The groove tapers and becomes shallow

toward the apex of the tooth and disappears before reaching the tip (Fig. 1*b*). Viewed in transverse (horizontal) section, it forms a deep invagination, which is lined by enamel (Fig. 1*c–d*). Together with the groove from the opposite side of the crown, it reduces the centre of the pulp cavity to a narrow slit for most of the tooth's height with the exception of the apical region (Fig. 1*d*). The anterior and posterior portions of the pulp cavity are distinct and more or less triangular in transverse section (Fig. 1*d*).

Each groove closely resembles the venom groove along the anterior edge of the poison fangs in many extant snakes, which forms a deep invagination². In some taxa (for example, Elapidae), this groove subsequently becomes closed by apposition of its margins to form a tube³. The pulp cavity becomes distorted during the formation of this tube and assumes a lunate outline in transverse section. The maxillary and dentary teeth in the two living species of the lizard *Heloderma* have a deep lingual groove for venom delivery along the anterior margin of the crown and often a less prominent furrow along the posterior margin as well^{4–5}. By analogy, the two grooves on the teeth of *Uatchitodon* presumably also served in venom conduction. The teeth of *Uatchitodon* differ from those of venomous squamate reptiles in the placement of the grooves on the labial and lingual surfaces of the crowns and in the presence of serrated cutting edges.

Features indicative of an oral venom delivery apparatus have previously only been reported in a single fossil amniote other than squamates. The therocephalian therapsid *Euchambesia* from the Upper Permian of South Africa has a deep lateral recess on the maxilla, which opens onto the palate behind the large canine. Grooves leading to the base of this tooth apparently transmitted products from a gland housed in the maxillary recess to a sulcus along the labial margin of the canine⁶. This represents the earliest example of an oral venom delivery apparatus in a tetrapod described to date.

The blade-like shape and the sharp, typically serrated cutting edges of the tooth crowns of *Uatchitodon* suggest a sectorial

function. The current lack of skeletal remains referable to *Uatchitodon* militates against specific palaeobiological inferences. Scenarios for the evolutionary origin of venom use in squamates have variously hypothesized oral toxins as a means to immobilize prey before ingestion, to aid in the digestion of the food bolus or to deter potential predators^{5,7-9}. There exists evidence to support each of these functions but venom use probably originally evolved in a feeding context and was only subsequently co-opted for defensive purposes^{5,9}.

The inferred venom grooves in *Uatchitodon* predate the earliest record of such features among squamates⁵ by over 100 million years. □

Received 17 January; accepted 6 March 1991.

1. Sues, H.-D. & Olsen, P. E. *Science* **249**, 1020–1023 (1990).
2. Peyer, B. *Comparative Odontology* (The University of Chicago Press, Chicago and London, 1968).
3. Bogert, C. M. *Bull. Am. Mus. nat. Hist.* **81**, 285–360 (1943).
4. Russell, F. E. & Bogert, C. M. *Toxicon* **19**, 341–359 (1981).
5. Pregill, G. K., Gauthier, J. A. & Greene, H. W. *Trans. San Diego Soc. nat. Hist.* **21**, 167–202 (1986).
6. Mendrez, C. in *Problèmes Actuels de Paléontologie: Évolution des Vertébrés*, Coll. intern. C.N.R.S. no. **218**, 379–408 (1975).
7. Gans, C. in *Biology of the Reptilia* Vol. 8: Physiology B (eds Gans, C. & Gans, K. A.) 1–42 (Academic, London/New York, 1978).
8. Thomas, R. G. & Pough, F. H. *Toxicon* **17**, 221–228 (1979).
9. Russell, F. E. in *The Structure, Development and Evolution of Reptiles* (ed. Ferguson, M. W. J.) 469–480 (Academic, London, 1984).

ACKNOWLEDGEMENTS. The work was supported by the National Science Foundation and National Geographic Society (H.-D.S. and P. E. Olsen). I thank P. A. Kroehler for technical assistance and V. Krantz for photographing the material.

Genetic evidence for the spread of agriculture in Europe by demic diffusion

Robert R. Sokal*, Neal L. Oden† & Chester Wilson*

* Department of Ecology and Evolution, State University of New York, Stony Brook, New York 11794-5245, USA

† Department of Preventive Medicine, Division of Epidemiology, Health Science Center, State University of New York, Stony Brook, New York 11794-8036, USA

EUROPEAN agriculture originated in the Near East about 9,000 years ago¹⁻³. The Neolithic reached almost all areas suitable for agriculture by 5,000 yr BP (before present)^{2,4}. The routes and times of the spread of agriculture through Europe are relatively well established^{2,3,5}, but not its manner of spreading⁶⁻⁸. This could have been by cultural diffusion with few genetic consequences. By contrast, Ammerman and Cavalli-Sforza² proposed that the spread of farming increased local population densities, causing demic expansion into new territory and diffusive gene flow between the neolithic farmers and mesolithic groups. We have now tested observed genetic patterns against expectations derived from the demic expansion hypothesis. We found significant partial correlations of genetic distances with a distance matrix especially designed to represent the spread of agriculture on that continent, when geographic distances are held constant. These findings support the hypothesis of Ammerman and Cavalli-Sforza and invite further investigation into Renfrew's hypothesis on the origin of the Indo-European languages.

From 3,373 locations in Europe, we sampled 26 genetic systems (red cell antigens, plasma proteins, enzymes, histocompatibility alleles, immunoglobulins; Table 1). Details are specified elsewhere⁹⁻¹¹. The number of localities ranged from 870 to 33 with a mean of 101. From these gene frequencies we computed Prevosti distances¹² separately for each genetic system, because different numbers of localities are available for each system.

A map of the onset of agriculture in Europe was obtained by pixel-by-pixel averages of spatially interpolated¹³ dates for latest

TABLE 1 Partial matrix correlation of genetic distances with origin-of-agriculture distances

System	$r_{(OOA, GEN), GEO}$	P
1.1 ABO	-0.16202	0.00000
1.2 A ₁ A ₂ BO	-0.02716	0.25240
2.5 MN	0.01793	0.68325
2.7 MN haplotypes	-0.06202	0.18177
3.1 P	-0.03435	0.23620
4.1 Rh	0.05448	0.99678
4.13 Rh haplotypes	-0.09464	0.04233
4.19 Rh haplotypes	-0.06856	0.11137
5.1 Lu	0.01491	0.55992
6.1 K	0.02275	0.70664
6.3 K (anti-K, -k)	-0.03673	0.32844
7.1 Se	-0.07997	0.13861
8.1 Fy	0.06725	0.92810
36.1 Hp	-0.04730	0.11253
37.1 Tf	0.23657	0.99834
38.1 Gc	-0.00321	0.39998
50.1.1 ACP1	0.21616	0.99962
52 PGD	0.01555	0.56605
53 PGM1	0.23784	0.99996
56 AK	-0.07062	0.13322
63 ADA	-0.02580	0.30443
65 T	-0.08506	0.05936
100 HLA-A	0.25741	0.99994
101-102 HLA-B	0.19968	0.99869
200 Gm 1,2,5	0.07732	0.81725
201 Km	-0.06247	0.27095

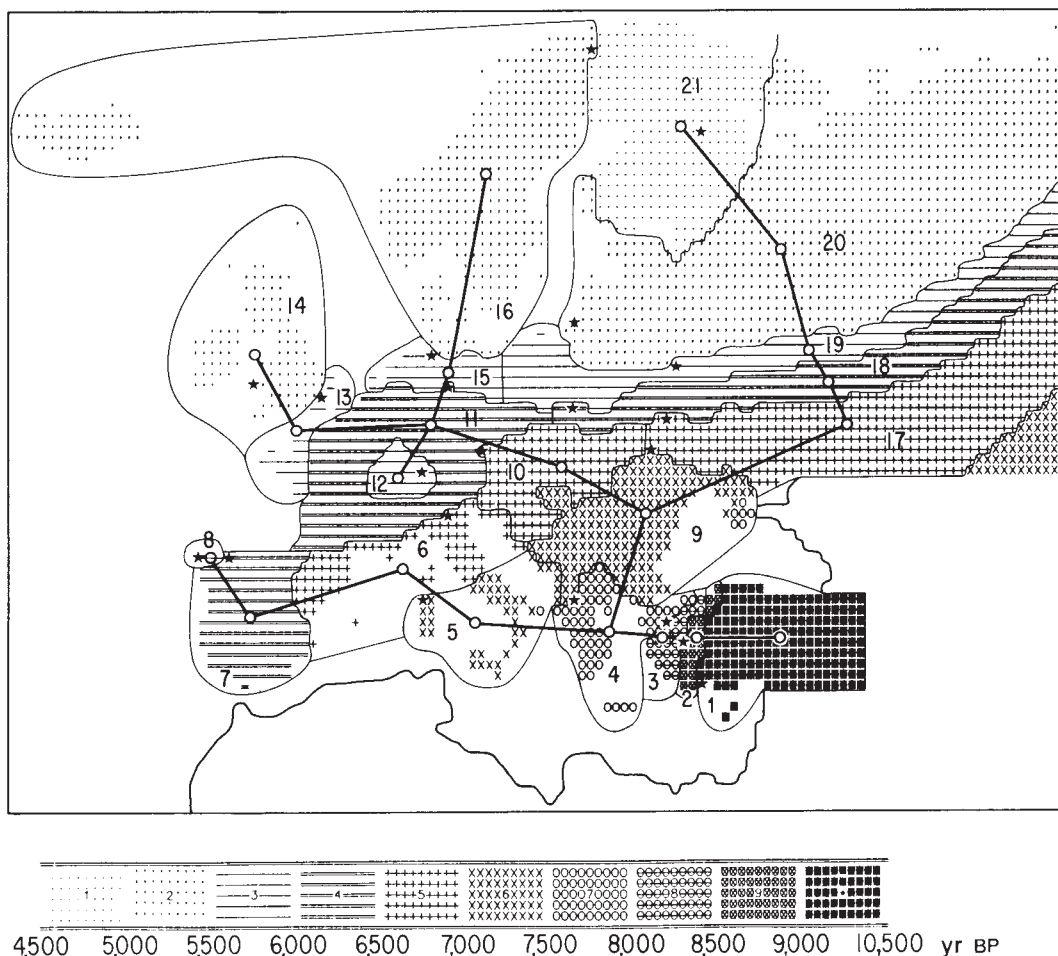
Geographic distances held constant. P is the left-tail cumulative probability based on the asymptotic approximation to normality. Thus all systems with $P \geq 0.95$ are significant at a one-tailed 5% level. Numbers preceding the system symbols, up to 65, are those assigned by Mourant *et al.*²⁸; those from 100 and above were assigned in our laboratory. r , Partial correlation coefficient.

observed mesolithic and earliest observed neolithic settlements² (Fig. 1). The resulting 500-year contour classes were divided into 21 regions that approximate successive stages in the spread of agriculture into and through Europe. The regions were connected by a directed graph also shown (Fig. 1). Each node of this graph was assigned the date of the latest pixel in its region. But for clarity, the nodes are shown in the figure in the centres of their regions. For a given set of localities sampled for any one system, we constructed an origin-of-agriculture (OOA) temporal distance matrix between all pairs of localities. The OOA distance between any two localities depends on whether their regions constitute different bifurcating branches of the OOA graph. If so, the OOA distance is the sum of the temporal distances from each point to the node of its preceding region plus the shortest temporal distance between the two nodes along the graph. If not, the points are either in the same region or in regions constituting a unidirectional path in the graph. In these cases, their OOA distance is simply their temporal distance in the map of Fig. 1. We also computed geographic distance (GEO) matrices as great circle distances between pairs of localities.

As determined by Mantel tests^{14,15}, genetic distances are related to geography¹⁶. Matrix correlations range from -0.034 (for Lutheran) to 0.588 (for HLA-B). Overall significance (by Fisher's method for combining probabilities¹⁷) is $P < 1.0 \times 10^{-6}$. Necessarily (Fig. 1), the OOA distances are substantially correlated with the GEO distances (range from 0.502 for Rh haplotypes of system 4.13, to 0.711 for Se; all correlations significant at $P < 5.0 \times 10^{-6}$). The genetic distance and OOA matrices are also significantly correlated (for 16 genetic systems, significant correlations range from 0.072 for ABO, to 0.520 for HLA-A). The overall significance is $P < 5.0 \times 10^{-6}$.

The correlation between genetics and OOA could be due to their common dependence on geography. We therefore tested the partial correlation of genetics with origin of agriculture while

FIG. 1 Contour map of the onset of agriculture. The contours represent 500-year intervals identified in the key. The area has been subdivided into 21 numbered 500-year regions in which the latest pixel is marked by a star. For further explanation, see text.



keeping geographic distances constant (Table 1). When tested for significance by the Smouse-Long-Sokal test¹⁸, six of the partial correlations (Rh system 4.1, Tf, ACP1, HLA-A, HLA-B, PGM1) remain significant at $P < 0.003$. Significance over all systems is very high ($P < 1.0 \times 10^{-6}$). There is clear evidence of a correlation between genetics and OOA above and beyond the predicted effects of geography¹⁹. Thus, our results support the demic diffusion hypothesis. Additional support comes from northwest-southeast clines predicted by Fisher's²⁰ model of a population wave of advance and demonstrated in a variety of ways^{11,21,22}.

Why should only 6 of 26 genetic systems show significant agreement with demic diffusion? Unless the allele frequencies of the immigrant farmers differed substantially from those of the native hunter-gatherers, there would be no trace of the migration and diffusion patterns. Sokal *et al.*²³ were unable to detect differences in gene frequency between immigrants and residents of less than 0.4 in simulated surfaces for 10,000 individuals. Although we cannot directly extend these findings to the human populations in our study, we note that only differences between major races show gene-frequency differentials of such a magnitude, and even then for only a few loci. When compared for over 60 alleles in Roychoudhury and Nei²⁴, Japanese differ in only two allele frequencies by 0.4 or more from those of Italians; Yorubas from Nigeria differed that much from Italians for only four alleles. But it is generally believed that the first agriculturalists in the Near East were members of the same major race (Caucasoids) as the mesolithic hunter-gatherers of Europe. Using modern Turks or Ashkenazi Jews as representative Near Easterners, we note that neither group differs by as much as 0.2 from the Italians (compared for over

45 and 50 allele frequencies, respectively). Thus, we should not be surprised at the low number of alleles supporting the demic expansion hypothesis.

The highly significant negative partial correlation for the ABO system in Table 1 clearly contradicts the demic diffusion hypothesis. The well known east-west cline of the B allele of that system^{25,26} runs perpendicularly to the spread of agriculture (Fig. 1). Not surprisingly, it shows negative fit to the OOA hypothesis.

We conclude that the spread of agriculture through Europe was not simply a case of cultural diffusion, but involved significant differential reproduction of the new farmers whose origins can be traced to the Near East. Support for the hypothesis of Ammerman and Cavalli-Sforza is a necessary first step towards testing Renfrew's hypothesis of the origin of Indo-European speakers in Europe²⁷, which equates the early Indo-Europeans with the early farmers. We are currently examining the genetic evidence in favour of Renfrew's hypothesis. □

Received 17 December 1990; accepted 4 March 1991.

1. Clark, J. G. D. *Proc. prehist. Soc.* **21**, 58-73 (1965).
2. Ammerman, A. J. & Cavalli-Sforza, L. L. *The Neolithic Transition and the Genetics of Populations in Europe* (Princeton University Press, Princeton, New Jersey, 1984).
3. Champion, T., Gamble, C., Shenan, S. & Whittle, A. *Prehistoric Europe* (Academic, London, 1984).
4. Barker, G. *Prehistoric Farming in Europe* (Cambridge University Press, Cambridge, 1985).
5. Barraclough, G. ed. *The Times Atlas of World History* (Hammond, Maplewood, New Jersey, 1979).
6. Zvelebil, M. & Zvelebil, K. V. *Antiquity* **62**, 574-583 (1988).
7. Ammerman, A. J. *Antiquity* **63**, 162-165 (1989).
8. Meiklejohn, C. & Zvelebil, M. in *Biocultural Approaches to Human Bones* (ed. Zvelebil, M.) (in the press).
9. Sokal, R. R., Oden, N. L. & Thomson, B. A. *Am. J. phys. Anthropol.* **76**, 337-361 (1987).
10. Derish, P. A. & Sokal, R. R. *Hum. Biol.* **60**, 801-824 (1988).
11. Sokal, R. R., Harding, R. M. & Oden, N. L. *Am. J. phys. Anthropol.* **80**, 267-294 (1989).
12. Prevosti, A., Ocana, J. & Alonso, G. *Theor. appl. Genet.* **45**, 231-241 (1975).

13. Dougenik, J. A. & Sheehan, D. E. *SYMAP User's Reference Manual, Version 5.20* (Camera Stat., Bedford, Massachusetts, 1979).
14. Mantel, N. *Canc. Res.* **27**, 209–220 (1967).
15. Sokal, R. R. *Syst. Zool.* **28**, 227–231 (1979).
16. Sokal, R. R. *Proc. natn. Acad. Sci. U.S.A.* **85**, 1722–1726 (1988).
17. Sokal, R. R. & Rohlf, F. J. *Biometry* 2nd Edn (Freeman, San Francisco, 1981).
18. Smouse, P. E., Long, J. C. & Sokal, R. R. *Syst. Zool.* **35**, 627–632 (1986).
19. Kimura, M. & Weiss, G. H. *Genetics* **49**, 561–576 (1964).
20. Fisher, R. A. *Ann. Eug.* **7**, 355–369 (1937).
21. Menozzi, P., Piazza, A. & Cavalli-Sforza, L. L. *Science* **201**, 786–792 (1978).
22. Sokal, R. R. & Menozzi, P. *Am. Nat.* **119**, 1–17 (1982).
23. Sokal, R. R., Jacquez, G. M. & Wooten, M. C. *Genetics* **121**, 845–855 (1989).

24. Roychoudhury, A. K. & Nei, M. *Human Polymorphic Genes, World Distribution* (Oxford University Press, Oxford, 1988).
25. Mourant, A. E. *Blood Relations: Blood Groups and Anthropology* (Oxford University Press, Oxford, 1983).
26. Sokal, R. R. *Hum. Biol.* **61**, 691–702 (1989).
27. Renfrew, C. *Archaeology and Language: The Puzzle of Indo-European Origins* (Jonathan Cape, London, 1987).
28. Mourant, A. E., Kopec, A. C. & Domaniewska-Sobczak, K. *The Distributions of the Human Blood Groups* (Oxford University Press, Oxford, 1976).

ACKNOWLEDGEMENTS. We thank B. Thomson, D. DiGiovanni, A. Mulhern and D. Cachia for technical assistance, and G. Barbujani for helpful comments. Part of the computation was carried out on the Cornell National Supercomputer Facility. This research was supported by the NSF (R.R.S.).

Direct activation of cardiac pacemaker channels by intracellular cyclic AMP

Dario DiFrancesco & Paolo Tortora

Università di Milano, Dipartimento di Fisiologia e Biochimica Generali, via Celoria 26, 20133 Milano, Italy

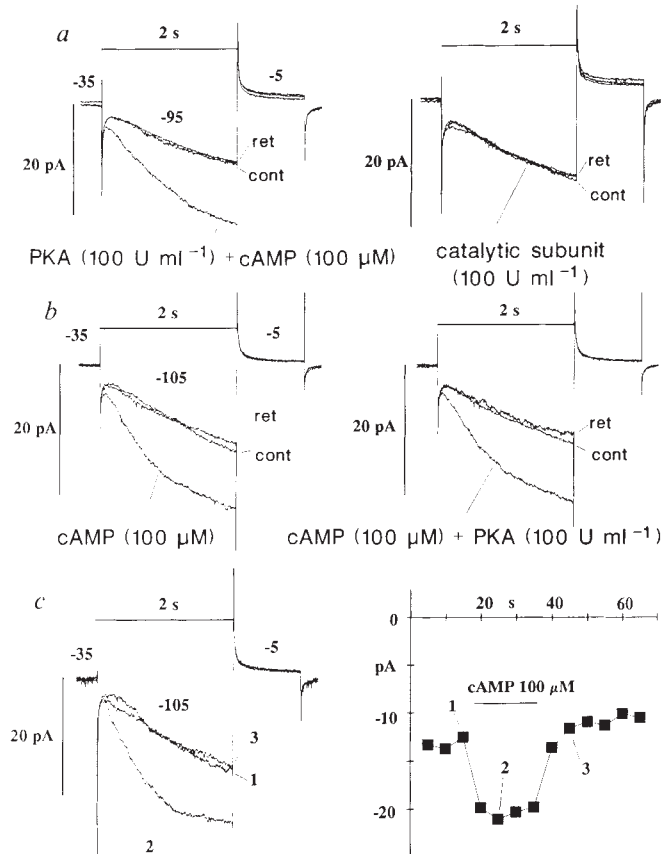
CYCLIC AMP acts as a second messenger in the modulation of several ion channels^{1–9} that are typically controlled by a phosphorylation process¹⁰. In cardiac pacemaker cells, adrenaline and acetylcholine regulate the hyperpolarization-activated current (i_f), but in opposite ways; this current is involved in the generation and modulation of pacemaker activity¹¹. These actions are mediated by cAMP and underlie control of spontaneous rate by neurotran-

smitters^{12–17}. Whether the cAMP modulation of i_f is mediated by channel phosphorylation is, however, still unknown. Here we investigate the action of cAMP on i_f in excised patches of cardiac pacemaker cells and find that cAMP activates i_f by a mechanism independent of phosphorylation, involving a direct interaction with the channels at their cytoplasmic side. Cyclic AMP activates i_f by shifting its activation curve to more positive voltages, in agreement with whole-cell results. This is the first evidence of an ion channel whose gating is dually regulated by voltage and direct cAMP binding.

The current i_f was recorded in cell-free inside-out macro-patches excised from the membrane of isolated rabbit sino-atrial node myocytes and containing hundreds of i_f channels¹⁸. In Fig. 1a (left) it can be seen that i_f recorded in one such patch during hyperpolarizations to -95 mV increases during perfusion of protein kinase A (PKA; 100 U ml⁻¹) with cAMP (100 μ M) at the intracellular side, in much the same way as i_f in whole-cell¹⁴ and single-channel measurements¹⁵ after β stimulation. By

FIG. 1 Current i_f recorded in inside-out macro-patches. The current i_f was activated by stepping to the voltages indicated at a frequency of 0.2 Hz. All voltages are conventionally expressed as differences between internal and external solutions. In a and b, control and test solutions used for perfusion of the internal side of the patch contained ATP (2 mM) and Mg^{2+} (2 mM). In c, control and test solutions did not contain any ATP or Mg^{2+} . In a and b, each trace is the average of $2/3$ recordings. a, Patch current records before (cont), during, and after (ret) exposure to a test solution containing protein kinase A (100 U ml⁻¹) plus cAMP (100 μ M) (left) and, subsequently, before, during, and after perfusion with the catalytic subunit of the PKA (100 U ml⁻¹) (right). Assays run before and after the experiment showed that the kinase activity had been fully retained (1.3×10^6 U per mg protein just after purification), indicating that the lack of action of the catalytic subunit was not due to loss of enzyme activity; b, similar protocol in another patch perfused in sequence with test solutions containing cAMP at 100 μ M (left) and cAMP (100 μ M) plus PKA (100 U ml⁻¹) (right); c, action of cAMP in the absence of ATP. Left, patch i_f records before (1), during (2), and after (3) perfusion with cAMP at 100 μ M; right, time-course of the i_f amplitude at -105 mV during cAMP perfusion.

METHODS. Single sino-atrial node myocytes were isolated from rabbit hearts as described²¹. Cells under study were allowed to settle in Petri dishes and superfused with a high- K^+ solution containing (mM): KCl, 130; NaCl, 10; $CaCl_2$, 2; EGTA, 5; HEPES-KOH, 10 (pH 7.4; pCa 7). Giga-seals were obtained with pipettes filled with a solution previously used for single i_f -channel recording¹⁵ and containing (mM): NaCl, 70; KCl, 70; $CaCl_2$, 1.8; $MgCl_2$, 1; $BaCl_2$, 1; $MnCl_2$, 2; HEPES-KOH, 5; pH 7.4. To overcome the difficulty of detecting single i_f channel currents which results from their low conductance (0.98 pS)¹⁵, large-tipped pipettes (about 1 – 3 μ m diameter with resistances in the range 0.5 – 2 M Ω) were used which allowed recordings from macro-patches generating currents of several pA¹⁸. The number of channels in a patch was calculated to be up to $\sim 1,000$, and typically in the range 200 – 300 , for the 111 patches considered here. After giga-seal formation, hyperpolarizations were applied in the cell-attached configuration from a holding potential of -35 mV (assuming a zero membrane-resting voltage in the high- K^+ solution used) to check for the presence of i_f channels. i_f records of several pA amplitude were frequently recorded under these conditions¹⁸. Patches giving successful recordings were pulled off the cell and perfused at the intracellular membrane side in the inside-out configuration, with control and test solutions delivered through a perfusion device that allowed sufficiently rapid (0.5 – 1 s) changes²¹. When patch excision led to vesicle formation, the pipette tip was briefly exposed to air to obtain the inside-out configuration. The perfusing control solution contained (mM): NaCl, 10; potassium aspartate, 130; $CaCl_2$, 2; EGTA, 5; HEPES-KOH, 10 (pH 7.2). ATP (Na^+ salt; 2 mM), $MgCl_2$ (2 mM) and GTP (0.1 mM) were present in some of the experiments. Tem-



perature was continuously monitored and was 26 – 28 $^{\circ}$ C. ATP, GTP, PKA and PKA inhibitor were from Sigma; GDP- β S from Boehringer-Mannheim; catalytic subunit of PKA from Sigma, or purified in our laboratory from bovine heart³⁰. Enzyme activity assayed after purification was 1.3×10^6 pmol min⁻¹ mg⁻¹ (1.3×10^6 U mg⁻¹), in agreement with previous values³⁰, and was abolished in the presence of the cAMP-dependent protein-kinase inhibitor (Sigma, type II); the purified enzyme ran as one band on an SDS-polyacrylamide gel with $M_r \sim 40$ K.

analogy with other cardiac channels controlled by cAMP^{3,7}, this effect could be due to phosphorylation of the channel protein. Unexpectedly, however, activation of i_f was not associated with a phosphorylation process, as indicated in Fig. 1a (right), in which perfusion of the same patch with the catalytic subunit of PKA (100 U ml⁻¹) did not result in current activation. In 17 out of 22 patches, perfusion with the holoenzyme (100 U ml⁻¹) and cAMP (100 μ M) resulted in i_f activation, whereas i_f activation was not observed in any of the 21 patches challenged with the catalytic subunit (activities in U ml⁻¹: 100, $n=4$; 500, $n=4$; 26,000, $n=7$; 36,400, $n=4$; 52,000, $n=2$). But perfusion with cAMP alone at 100 μ M did stimulate the current i_f , as shown in Fig. 1b (left). The action of cAMP was not modified by addition of the holoenzyme (Fig. 1b, right), indicating that the i_f increase was attributable only to the cyclic nucleotide. We obtained i_f activation by cAMP in the presence of ATP in 26

patches. In 4 of these patches, PKA at 100 U ml⁻¹ did not alter the i_f stimulation induced by 100 μ M cAMP.

Activation of i_f by cAMP was not a result of phosphorylation by endogenous protein kinases, because it could be reproduced after removal of ATP and Mg²⁺ from the intracellular solution (Fig. 1c), and after addition of a non-specific protein-kinase inhibitor (H7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine; Sigma) at 10 μ M, $n=4$; 100 μ M, $n=2$) or of a specific PKA inhibitor (Sigma peptide inhibitor, rabbit sequence; 0.1 μ M, $n=4$). The action of cAMP had a rapid onset (less than 5 s) and was also rapidly reversed (Fig. 1c, right). Stimulation of i_f by cAMP in the absence of ATP and Mg²⁺ was obtained in 61 out of 67 patches. Although phosphorylation-dependent steps may exist elsewhere in the modulation cascade¹⁹, these data indicate that cAMP action is independent of phosphorylation.

It has been suggested that i_f channels may be directly coupled to G proteins¹⁸. We have excluded the possibility that cAMP action on i_f is mediated by G proteins, because it was still present in the absence of GTP ($n=24$) or when GTP was replaced by guanosine-5'-O-(2-thiodiphosphate) (GDP- β S; 100 μ M) as an inhibitor of G-protein function²⁰ ($n=5$).

In intact nodal cells increasing or decreasing intracellular cAMP does not alter the fully activated i_f , but shifts its activation curve to more positive or negative voltages^{14,21}. So β stimulation increases the probability of single i_f channel opening, without changing the channel conductance¹⁵. Figure 2 shows that cAMP acts similarly on patch i_f . In Fig. 2A, cAMP at 100 μ M increases i_f at -115 mV and decreases it at -155 mV, as expected for a positive shift of the activation curve²¹, without modifying the fully activated current value at the latter voltage. In the more complete analysis of Fig. 2B, cAMP at 30 μ M did not affect the fully activated I/V relation in the range -155/-25 mV (panel c), although it accelerated current activation and slowed current deactivation (panels a and b). Similar results were obtained in another cell. These results agree with the action of cAMP on i_f in whole-cell experiments^{14,16,21}.

Cyclic AMP-induced shifts of the i_f activation curve were measured¹⁷ for different cAMP concentrations and the dose-response relationship is plotted in Fig. 3. Hill-plot fitting yielded values of 0.211 μ M for the half-maximal cAMP concentration and 0.850 for the Hill coefficient. Cyclic AMP concentrations involved in the modulation of i_f gating are well within the range controlled under physiological conditions by adenylyl cyclase (0.2–5 μ M in cardiac tissue)²². The maximal cAMP-induced shift of 11 mV is slightly larger than that caused by isoprenaline

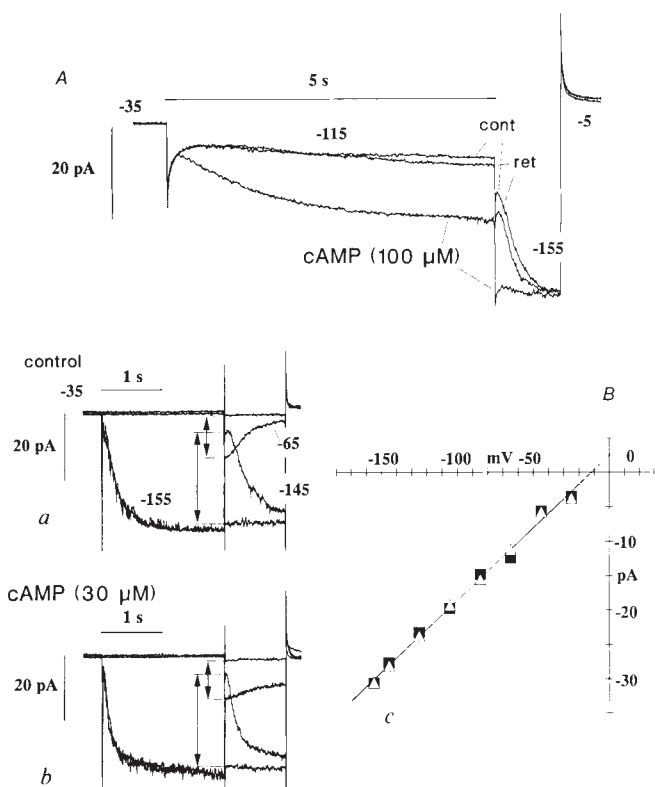


FIG. 2 A, Protocol applied every 10 s to an inside-out macro-patch before (cont), during, and after (ret) perfusion of the internal membrane side with cAMP at 100 μ M. All solutions were ATP- and GTP-free. In the control solution, stepping to -115 mV activated a fraction of the available i_f channels, and the following step to -155 mV activated the remaining fraction. During perfusion with cAMP, the fraction of current activated at -115 mV increased, while that at -155 mV decreased. This indicates that cAMP displaced the i_f activation curve to more positive voltages without alteration of the fully activated current at -155 mV. B, Lack of action of cAMP on the i_f fully activated I/V relation. a and b, Patch i_f records in a control (ATP- and GTP-free) solution (a) and after addition of cAMP at 30 μ M (b). The procedure has been described¹⁴ and consisted of paired clamp protocols in which steps to test voltages were either delivered from the holding potential of -35 mV, or were preceded by a fully activating pulse to -155 mV for 2 s. The I/V curve was then measured as the amplitude of the difference current at each test potential (arrows). Paired records at -65 and -145 mV are shown in both control (a) and cAMP (b) solutions. All traces were digitally corrected for linear leakage and capacitive transients. c, Fully activated I/V relation for patch i_f in the absence (■) and in the presence (△) of cAMP at 30 μ M. Best-fitting linear relations (full lines) have reversal potentials of -11.5 mV (control) and -12.4 mV (cAMP) and slope conductances of 209 pS (control) and 211 pS (cAMP). From these values and the known single i_f channel conductance¹⁵, the channels in this patch were about 213–215.

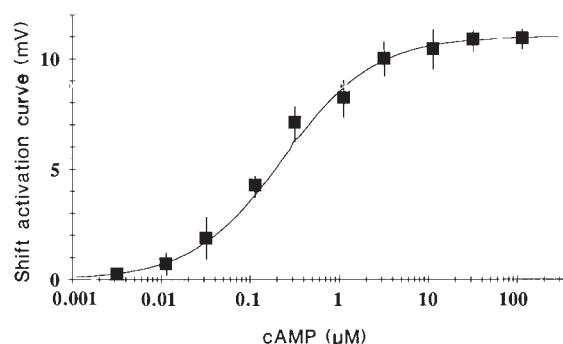


FIG. 3 Dose-response relationship for the shift of i_f activation curve as a function of cAMP concentration from 35 patches. Shifts were measured as described¹⁷. Mean $\pm$ s.e.m. values are plotted. The numbers of patches tested at the various concentrations (μ M) were: 4 (0.003), 4 (0.01), 4 (0.03), 4 (0.1), 8 (0.3), 7 (1), 6 (3), 6 (10), 9 (30) and 13 (100). Data were best fitted using the Hill equation $y/y_{\max} = 1/(1 + (k_{1/2}/c)^n)$, where y is the measured shift, $y_{\max}$ is the shift at saturating concentrations (set to 11 mV) and c is the cAMP concentration (full line). This yielded a half-maximal concentration $k_{1/2} = 0.211$ μ M and a Hill factor $n = 0.850$.

(1 μ M) in whole cells (7.84 ± 0.72 mV; $n = 11$), as is to be expected if a basal level of cAMP is present in sino-atrial node myocytes¹⁶.

The i_f was also activated, although with a reduced specificity, by cGMP and cCMP, which we tested for comparison with other cyclic nucleotide-sensitive conductances in receptor cells²³⁻²⁶. We measured shifts of 1.10 ± 0.66 ($n = 4$), 5.27 ± 0.08 ($n = 3$) and 9.63 ± 0.49 ($n = 8$) mV with cGMP at 0.3, 10 and 100 μ M, respectively; and 2.59 ± 0.47 ($n = 14$), 5.87 ± 0.41 ($n = 17$) and 6.24 ± 0.98 ($n = 18$) mV with cCMP at 10, 100 and 1,000 μ M, respectively. Apparent dissociation constants and Hill coefficients, assuming maximal shifts of 11 mV (for cGMP) and 6.5 mV (for cCMP), were $k_{1/2} = 7.85$ μ M and $n = 0.704$ (cGMP), and $k_{1/2} = 11.85$ μ M and $n = 0.784$ (cCMP).

By modulating i_f and other (K^+ and Ca^{2+}) currents, cAMP plays an essential part in the control of electrical and contractile activity of cardiac myocytes. Although cardiac Ca^{2+} and K^+ channels^{3,7}, as well as several other channels in different tissues¹⁰, are modulated by cAMP by phosphorylation, our data indicate that the action of cAMP on i_f is exerted by direct interaction with the i_f channels.

Direct channel regulation by cyclic nucleotides has been demonstrated in various receptor cells²³⁻²⁶, of which only olfactory receptors are sensitive to cAMP²⁶. Although, interestingly, in all cases non-specific cation conductances like i_f are controlled by cyclic nucleotides, the i_f modulation by cAMP reported here is different in that (1) i_f channels are voltage-gated; (2) sino-atrial node cells are non-receptor cells; (3) i_f channels are more sensitive to cAMP than to cGMP; (4) the Hill coefficient of i_f modulation by cAMP (0.824) is lower than that in sensory channels^{26,27} and suggests a 1:1 binding.

I_f channel modulation by cAMP in the sino-atrial node cell underlies neurotransmitter control of the cell spontaneous activity and of cardiac rhythm. The presence of a direct, phosphorylation-independent control of i_f channel gating provides a rapid and efficient mode of channel control not requiring metabolic input.

Several types of nerve cells possess i_f -like currents¹¹, for some of which cAMP-dependent stimulation has been reported^{28,29}. It will be interesting to know if cAMP directly regulates i_f -like currents in these preparations also. $\square$

Immunomodulation of experimental allergic encephalomyelitis by antibodies to the antigen-Ia complex

Rina Aharoni, Dvora Teitelbaum, Ruth Arnon* & Joseph Puri

Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

AUTOIMMUNE diseases occur when T lymphocytes become activated on recognizing self antigen linked to the autologous class II molecule of the major histocompatibility complex (MHC)^{1,2}. The resulting complex of antigen MHC T-cell receptor could be a target for treatment of autoimmune diseases. Studies in which each component is blocked separately³⁻¹⁰ might be limited by interference in non-relevant immune responses that either use the same set of T-cell-receptor V gene segments or are linked to the same MHC. We report here an attack by a specific antibody on the unique antigenic site formed by the binding of two components of the trimolecular complex, the autoantigen bound to the self MHC¹¹⁻¹⁴. We tested its effect in experimental allergic encephalomyelitis, an acute neurological autoimmune disease which is widely regarded as a model for autoimmune disorders¹⁵⁻¹⁷ and which is mediated by CD4⁺ T cells recognizing myelin basic protein (BP), or its peptides, in association with self Ia^{18,19}. We made monoclonal antibodies which bound only the complex of BP and I-A^s. These antibodies blocked the proliferative response *in vitro* to the encephalitogenic determinant of BP and reduced the response to intact BP, without affecting the response to a nonrelevant antigen-purified protein derivative of tuberculin presented on syngeneic macrophages. They also inhibited experimental allergic encephalomyelitis in H-2^s mice. Hence, antibodies directed specifically to the autoantigen-Ia complex, may offer a highly selective and effective treatment in autoimmune diseases.

To obtain anti-BP/Ia^s monoclonal antibodies, B₁₀D₂ mice were immunized with glutaraldehyde-fixed SJL/J macrophages that had been pulsed with rat basic protein (RBP) and their spleen cells fused with the NS0 plasmacytoma line. The hybridomas obtained were screened for their ability to block selectively the proliferation of three different effector T-cell clones specific to: (1) the same antigen and the same Ia of the immunizing cells (SJL-RBP-6); (2) another antigen-purified protein derivative of tuberculin on the same H-2 haplotype (SJL-PPD-5); and (3) RBP on a different H-2 haplotype (B₁₀PL-RBP-20). The results for representative hybridomas are summarized in Table 1. Out of 800 hybridomas tested, 778 had no effect, as represented by clone C-25; five lines (for example A-6) inhibited the responses of all the clones tested non-specifically; seven hybridomas, represented by C-11, were specific to Ia^s and inhibited only the H-2^s T-cell clones; and six other lines (for example B-10) recognized the antigen BP and therefore inhibited all BP-specific clones. Only four hybridomas, (A-10-12, B-7-1, B-18-7 and C-34-72) inhibited exclusively the proliferative response of the SJL-RBP-6 encephalitogenic clone, with little or no effect on the two non-relevant clones. These antibodies were tested for specificity to the BP/H-2^s complex.

The ability of these antibodies (all IgM by isotype analysis), to bind specifically to SJL/J peritoneal macrophages pulsed with RBP was demonstrated both by fluorescence-activated cell sorting (FACS) analysis and complement-mediated cytotoxicity assay. FACS analysis showed that $44.3 \pm 3.0\%$ and $43.2 \pm 2.8\%$

Received 26 October 1990; accepted 4 March 1991.

1. Tsien, R. W. *Advances in Cyclic Nucleotide Research* **8**, 363-420 (1977).
2. Reuter, H. *Nature* **301**, 569-574 (1983).
3. Osterrieder, W. *et al. Nature* **298**, 576-578 (1982).
4. de Peyer, J. E., Cachelin, A. B., Levitan, I. B. & Reuter, H. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4207-4211 (1982).
5. Shuster, M. J., Camardo, J. S., Siegelbaum, S. A. & Kandel, E. R. *Nature* **313**, 392-395 (1985).
6. Ewald, D. A., Williams, A. & Levitan, I. B. *Nature* **315**, 503-506 (1985).
7. Walsh, K. B. & Kass, R. S. *Science* **242**, 67-69 (1988).
8. Li, M. *et al. Nature* **331**, 358-360 (1988).
9. Kume, H., Takai, A., Tokuno, H. & Tomita, T. *Nature* **341**, 152-154 (1989).
10. Levitan, I. B. *J. Memb. Biol.* **87**, 177-190 (1985).
11. DiFrancesco, D. *Prog. Biophys. molec. Biol.* **6**, 163-183 (1985).
12. Tsien, R. W. *J. gen. Physiol.* **64**, 293-319 (1974).
13. Brown, H. F., DiFrancesco, D. & Noble, S. J. *Nature* **280**, 235-236 (1979).
14. DiFrancesco, D., Ferroni, A., Mazzanti, M. & Tromba, C. *J. Physiol., Lond.* **377**, 61-88 (1986).
15. DiFrancesco, D. *Nature* **324**, 470-473 (1986).
16. DiFrancesco, D. & Tromba, C. *J. Physiol., Lond.* **405**, 493-510 (1988).
17. DiFrancesco, D., Ducouret, P. & Robinson, R. B. *Science* **243**, 669-671 (1989).
18. Yatani, A., Okabe, K., Codina, J., Birnbaumer, A. & Brown, A. M. *Science* **249**, 1163-1166 (1990).
19. Chang, F., Rosen, M., Tromba, C., Cohen, I. S. & DiFrancesco, D. *Biophys. J.* **57**, 141a (1990).
20. Eckstein, F., Cassel, D., Levkovitz, H., Lowe, M. & Selinger, Z. *J. biol. Chem.* **254**, 9829-9834 (1979).
21. DiFrancesco, D. & Tromba, C. *J. Physiol., Lond.* **405**, 477-491 (1988).
22. Teresaki, W. L. & Brooker, G. *J. biol. Chem.* **252**, 1041-1050 (1977).
23. Fesenko, E. E., Kolesnikov, S. S. & Lyubarsky, A. L. *Nature* **313**, 310-313 (1984).
24. Haynes, L. W. & Yau, K. W. *Nature* **317**, 61-64 (1986).
25. Johnson, E. C. *et al. Nature* **324**, 468-470 (1986).
26. Nakamura, T. & Gold, G. H. *Nature* **325**, 442-444 (1987).
27. Dhallan, R. S., Yau, K., Schrader, K. & Reed, R. R. *Nature* **347**, 184-187 (1990).
28. Bobker, D. & Williams, J. T. *Neuron* **2**, 1535-1540 (1989).
29. Pape, H.-C. & McCormick, D. A. *Nature* **340**, 715-718 (1989).
30. Demaille, J. G., Peters, K. A. & Fischer, E. H. *Biochemistry* **16**, 3080-3086 (1977).

ACKNOWLEDGEMENTS. This work was supported by the CNR and by the Ministry of University and Scientific Research. We thank G. Maccaferri, M. Mangoni, L. Cattin, T. Tritella for assistance and I. S. Cohen for discussion.

* To whom correspondence should be addressed.

of RBP-pulsed SJL/J macrophages were stained by antibodies B-18-7 and C-34-72, respectively, in comparison with $32.6 \pm 0.1\%$ and $30.3 \pm 0.7\%$ binding to unpulsed macrophages. Only $29.4 \pm 0.9\%$ of the RBP-pulsed SJL/J cells were stained by non-relevant IgM control antibody (anti ricin). There was similar staining, $30.4 \pm 1.1\%$ and $32.1 \pm 0.6\%$, with RBP-pulsed macrophages of BALB/c (H-2^d) mice in the presence of B-18-7 and C-34-72 antibodies, respectively. Thus 10–15% specific binding has been demonstrated. These results were reproducible in three independent experiments using either culture supernatant or ascitic fluid. In competition experiments (data not shown) the B-18-7 and C-34-72 antibodies consistently inhibited, though to a very limited degree (about 3%), the binding of anti-I-A^s serum to RBP-pulsed macrophages. This may be because only a small proportion of I-A molecules are occupied by the processed antigen²¹, or because of the low affinity of these antibodies in comparison with the anti-I-A^s serum. A functional assay of complement-mediated cytotoxicity showed (Fig. 1A) specific lysis by antibodies B-7-1, B18-7 and C-34-72 only when RBP or peptide 89-101 (the relevant encephalitogenic determinant

in H-2^s mice^{22,23}) were presented on SJL/J but not on BALB/c macrophages. These antibodies did not lyse a non-relevant antigen, lysozyme, even when presented on SJL/J haplotype, nor did they lyse untreated SJL/J macrophages. The results (11.4–15.9% specific lysis) were comparable with those from the FACS analysis, and to other published results²¹. These antibodies did not bind to free RBP or to peptide 89-101 in a solid-phase radioimmunoassay (data not shown). These data indicate that these antibodies recognize only the 89-101/Ia^s complex.

We then tested the effect of the anti-BP/Ia^s antibodies on the T-cell response of mice that had been treated with the antibodies after immunization with RBP (Fig. 1B). The antibodies completely blocked the T-cell response of the SJL/J mice to the encephalitogenic determinant, and reduced the response to the intact BP from either rat (RBP) or mouse (MBP) (Fig. 1B, a). This inhibition was specific, as the response to PPD was not effected. The anti-complex antibodies inhibited only the response of H-2^s mice, SJL/J and B10S (Fig. 1B, a and b), but no inhibition was observed in SWR (H-2^d) and B10PL (H-2^u) mice (Fig. 1B, c and d). The humoral response of H-2^s mice

FIG. 1 A, Specificity of monoclonal antibodies to antigen/Ia complex, complement mediated cytotoxicity on antigen-pulsed macrophages. B, Anti-antigen/Ia effect on the T-cell response of mice from various strains immunized with RBP. METHODS. A, Peritoneal macrophages (1×10^6 cells per ml), were cultured for 18 h in medium supplemented with 200 units ml^{-1} murine interferon- γ and $100 \mu\text{Ci ml}^{-1}$ $^{51}\text{CrO}_4$. During the last 2 h of culture, cells were pulsed with either RBP (10 mg ml^{-1}), peptide 89-101 (1 mg ml^{-1}) or lysozyme (10 mg ml^{-1}). The target cells were then incubated with hybridoma supernatants ($50 \mu\text{l}$) and 1:10 diluted rabbit complement (from fresh serum of young rabbit). After 1 h supernatant samples were collected and ^{51}Cr release was determined. The experiment was repeated four times. The bar graphs plot the mean c.p.m. of triplicate samples after subtraction of the spontaneous release obtained by mixing target cells with media alone for the duration of the assay (327 ± 31). Error bars indicate standard deviation. Maximal release after total Nonidet P-40 lysis was $37,109 \pm 4,295$ and $30,737 \pm 3,957$ for SJL/J and BALB/c, respectively. The specific lysis obtained by the anti-complex antibodies was 11.4–15.9%. These values were calculated as follows: % specific lysis = [(experimental – unpulsed control)/(total – unpulsed control)] $\times 100$. B, Mice were immunized with RBP ($400 \mu\text{g}$ per mouse) on day 0, in complete Freund's adjuvant supplemented with 4 mg ml^{-1} H37Ra mycobacterium tuberculosis, followed by i.v. injections of pertussis toxin immediately and 48 h later. Ascitic fluids (0.5 ml), of anti-antigen/Ia antibodies, or NSO ascities which contained predominantly IgM isotype, were injected into the peritoneum on days -2, -1, 0, +1, +2, +3, +5, +7, +9. Lymph nodes and spleen cells were taken after 10 days and tested in proliferation assay, using 5×10^5 cells and the following antigens: RBP ($10 \mu\text{g}$), MBP ($10 \mu\text{g}$), P89-101 ($1 \mu\text{g}$), P1-11 ($1 \mu\text{g}$), for 4 days. The experiment was performed four times. Results are expressed as the mean c.p.m. of triplicates $\pm$ s.d. after subtracting the thymidine incorporation of cells incubated without antigen.

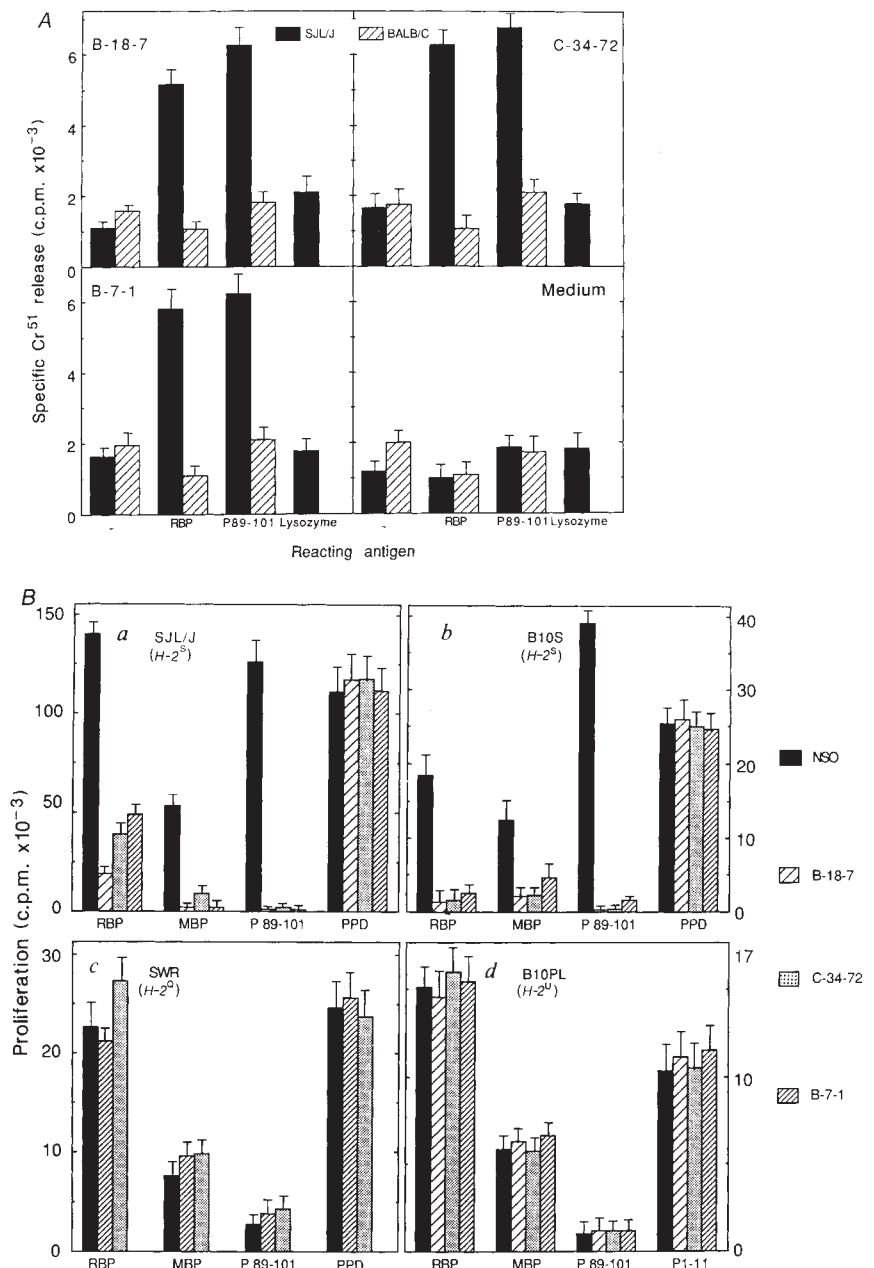


TABLE 1 Inhibition of T-cell proliferation by representative monoclonal antibodies

Inhibitor	Proliferation, Δ c.p.m. (inhibition, %)					
	SJL-RBP-6		SJL-PPD-5		B10.PL-RBP-20	
Medium	28,752	(0%)	26,196	(0%)	53,776	(0%)
C-25	29,951	(0%)	26,003	(1%)	54,169	(0%)
A-6	3,165	(89%)	8,648	(66%)	11,813	(78%)
C-11	17,629	(39%)	17,831	(32%)	52,172	(3%)
B-10	12,078	(58%)	24,309	(7%)	19,143	(64%)
A-10-12	15,483	(46%)	24,404	(7%)	51,949	(4%)
B-7-1	521	(98%)	26,254	(0%)	44,257	(18%)
B-18-7	132	(100%)	25,304	(4%)	51,483	(4%)
C-34-72	764	(97%)	27,001	(0%)	48,401	(10%)

BP-Ia^s complexes for immunization were prepared by *in vitro* pulsing of SJL/J spleen cells with $10 \mu\text{g ml}^{-1}$ RBP for 8 h. RBP had been prepared as described previously²³. Cells were then washed three times and fixed with 1% glutaraldehyde. The antigen-pulsed spleen cells were injected in complete Freund's adjuvant to the footpads of B₁₀D₂ mice (100×10^6 per mouse). Three more intraperitoneal injections of pulsed macrophages in PBS were given at two-week intervals. Four days after the last injection, spleen cells from immunized mice were fused with NSo plasmacytoma cells in a ratio of 4:1, respectively, according to Eshhar *et al.*²⁴. Screening was performed by adding $50 \mu\text{l}$ of hybridoma culture supernatants to 2.5×10^4 T cells, 5×10^5 irradiated spleen cells and $2.5 \mu\text{g}$ per well of antigen. After 48 h, the cultures were pulsed with [³H]thymidine and cells were collected 16 h later. The mean c.p.m. [³H] thymidine incorporation was calculated for triplicate cultures. Standard deviations for triplicate cultures were within 20% of the mean. Incorporation of the controls without antigen was less than 1,000 c.p.m.

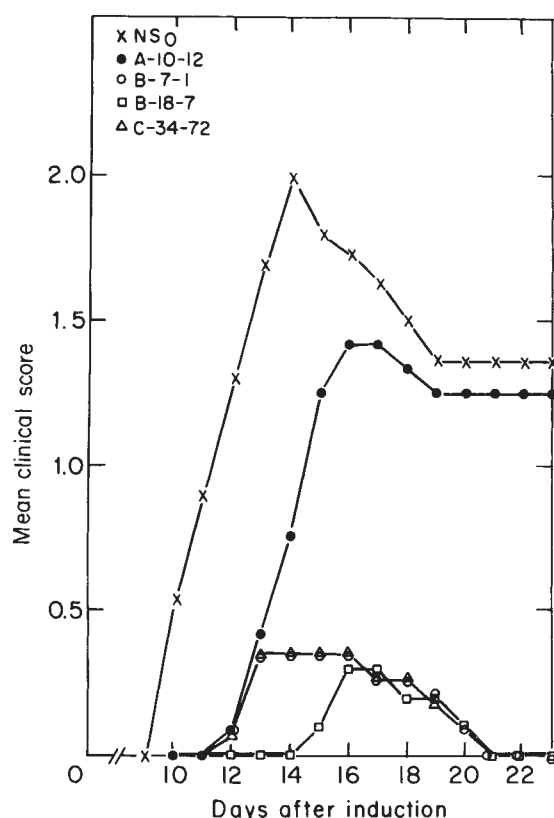


FIG. 2 Inhibition of EAE by anti-antigen/Ia complex antibodies in SJL/J mice. METHODS. EAE was induced on day 0, by injecting 5 mg lyophilized mouse spinal cord homogenate in enriched CFA followed by pertussis toxin as described in Fig. 1B. Ascitic fluids of monoclonal antibodies (0.5 ml) were injected into the peritoneum on days -2, -1, 0, +1, +2, +3, +5, +7, +9. After 10 days mice were examined daily for signs of EAE and assessed for clinical severity as follows: 1, flaccid tail; 2, hind limbs paralysis; 3, hind and fore limbs paralysis; 4, total paralysis; 5, moribund. Each point represents daily mean clinical score of 10-12 mice.

TABLE 2 Inhibition of EAE by anti-antigen/Ia complex antibodies in mice of various strains

Strain of mouse	NSo		Clinical score 0-5	B-18-7		Clinical score 0-5
	Incidence of disease Clin.	Hist.		Incidence of disease Clin.	Hist.	
SJL/J	8/11 (73%)	5/6 (83%)	2.2	1/10 (10%)	0/5 (0%)	0.3
H-2 ^s	2/7* (28%)	5/7* (71%)	0.3*	0/10* (0%)	0/10* (0%)	0*
ASW	3/7 (42%)	3/7 (42%)	0.6	0/13 (0%)	0/13 (0%)	0
H-2 ^s	7/13 (53%)	5/6 (69%)	1.1	9/13 (83%)	5/6 (83%)	2.5
SWR	5/10 (50%)	4/6 (66%)	0.7	5/10 (50%)	4/6 (66%)	0.9
H-2 ^u						

EAE was induced on day 0 by immunization with either spinal cord homogenate, as described in Fig. 2, or by RBP, 400 μg per mouse (entries indicated by an asterisk). Inhibition was obtained by intraperitoneal injection of 0.5 ml of the tested ascites on days -2, -1, 0, +1, +2, +3, +5, +7, +9. Clin., clinical; Hist., histological.

was also inhibited by the anti-complex antibodies (data not shown). The anti-BP/Ia^s antibodies reduced antibody formation to BP, and removed the response to peptide 89-101, but had no effect on the antibody response to PPD. These findings are indicative of the role of the T helper cells, which also recognize the antigen when bound to Ia, in the antibody response to BP.

The anti-BP/Ia^s antibodies were also tested for their capacity to block the clinical and histological manifestations of experimental allergic encephalomyelitis (EAE) (Fig. 2). Three clones, B-7-1, B-18-7 and C-34-72, caused inhibition of the disease in SJL/J mice in comparison with control ascitic fluid of the NSo plasmacytoma line. The hybridoma A-10-12, which was less active *in vitro* (Table 1), was also less efficient in the inhibition of EAE induction *in vivo*. The inhibition was specific to the H-2^s haplotype as it was also observed in ASW mice (Table 2), whereas in other EAE-sensitive strains of different haplotype, for example in SWR (H-2^u) and B₁₀PL (H-2^u) there was no effect. The anti-BP/Ia^s antibodies inhibited EAE induced by both spinal cord homogenate and purified BP.

These results suggest that by immunizing against the autoantigen presented by self MHC, specific anti-complex antibodies that bind only to the specific antigen in the right MHC context can be obtained. Such antibodies suppress specifically the pathogenic process and not other T-cell responses linked to the same MHC. These antibodies do not exert an adverse effect on myelin, as they cannot recognize the MBP in the target organ and thus offer a highly selective and effective treatment to EAE. This approach might be applied to the human analogue of EAE, namely multiple sclerosis, and to other autoimmune diseases. Even though macrophages pulsed with the whole BP were used for immunization, antibodies which specifically recognize the encephalitogenic peptide 89-101 presented on the Ia^s were selected by using a pathogenic T-cell clone for screening. Thus, it is not essential to identify the disease-inducing determinant in order to apply this strategy in other systems where exact knowledge of the autoantigen is not yet available. □

Received 12 December 1990; accepted 8 March 1991.

- Todd, J. A. *et al.* *Science* **240**, 1003-1009 (1988).
- Hohlfeld, R. *Ann. Neurol.* **25**, 532-538 (1989).
- Ben-Nun, A., Wekerle, H. & Cohen, I. R. *Nature* **292**, 60-61 (1981).
- Acha-Orbea, H. *et al.* *Cell* **54**, 263-273 (1988).
- Vandenbark, A. A., Hashim, G. & Offner, H. *Nature* **341**, 541-544 (1989).
- Howell, M. D. *et al.* *Science* **246**, 668-670 (1989).
- Steinman, L., Rosenbaum, J. T., Sriram, S. & McDevitt, H. O. *Proc. natn. Acad. Sci. U.S.A.* **78**, 7111-7114 (1981).

8. Sriram, S., Topham, D. J. & Carroll, L. *J. Immun.* **139**, 1485-1489 (1987).
9. Wraith, D. C., Smilek, D. E., Mitchell, D. J., Steinman, L. & McDevitt, H. O. *Cell* **59**, 247-255 (1989).
10. Urban, J. L., Horvath, S. J. & Hood, L. *Cell* **59**, 257-271 (1989).
11. Puri, J. & Lönai, P. *Eur. J. Immun.* **10**, 273-281 (1980).
12. Babbitt, B. P., Allen, P. M., Matsueda, G., Heber, E. & Unanue, E. R. *Nature* **317**, 359-361 (1985).
13. Buus, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 3968-3971 (1986).
14. Brown, J. H. *et al. Nature* **332**, 845-850 (1988).
15. Paterson, P. Y. in *Autoimmunity* (ed. Tala, N.) 644-692 (Academic, New York, 1977).
16. Fritz, R. B. & McFarlin, D. E. *Chem. Immun.* **46**, 101-125 (1989).
17. Zamvil, S. S. & Steinman, L. A. *Rev. Immun.* **8**, 579-621 (1990).
18. Offner, H., Brostoff, S. W. & Vardenbark, A. A. *Cell Immun.* **100**, 364-373 (1986).
19. Fritz, R. H., Skeen, M. J., Jen-Chou, C. H., Garcia, M. & Egorov, I. K. *J. Immun.* **134**, 2328-2332 (1985).
20. Demetz, S., Grey, H. M. & Sette, A. in *Proc. 7th Int. Cong. Immun.* (eds Melchers, F. *et al.*) 60-67 (Springer, Berlin, 1989).
21. Zamvil, S. S. *et al. J. exp. Med.* **168**, 1181-1186 (1988).
22. Sakai, K. *et al. J. Neuroimmun.* **19**, 21-32 (1988).
23. Hirshfeld, H., Teitelbaum, D., Arnon, R. & Sela, M., *FEBS Lett.* **7**, 317-320 (1970).
24. Eshhar, Z., Ofarim, M. & Waks, T. *J. Immun.* **124**, 775-780 (1980).

ACKNOWLEDGEMENTS. We thank P. Lönai for his support through this study, A. Meshorer for the histological evaluations and I. Cohen for reviewing the manuscript. This research was supported by Lilly Schilling, Yeda and Wolf Foundations.

Clonal deletion of immature CD4⁺CD8⁺ thymocytes in suspension culture by extrathymic antigen-presenting cells

Wojciech Swat, Leszek Ignatowicz,
Harald von Boehmer* & Pawel Kisielow†

Institute of Immunology and Experimental Therapy,
Polish Academy of Sciences, ul. Czerska 12, 53-114 Wrocław, Poland

* Basel Institute for Immunology, 487 Grenzacherstrasse, CH-4005,
Basel, Switzerland

† To whom correspondence should be addressed

ONE mechanism ensuring self tolerance of T cells is the clonal deletion of thymocytes bearing $\alpha\beta$ T-cell receptors¹⁻⁴. The stage of thymocyte development at which the interaction with antigen-presenting cells (APCs) leads to deletion, however, has not been determined directly. Indirect evidence suggests that intrathymic APCs induce deletion of CD4⁺CD8⁺ thymocytes³⁻⁶ (which die by

apoptosis⁷) but deletion at less⁸ and more mature⁹ developmental stages has also been implied. It is also not clear if clonal elimination of thymocytes can be triggered by peripheral antigens carried on extrathymic APCs migrating through the thymus¹⁰. Here we show antigen-specific induction of apoptosis in CD4⁺CD8⁺ thymocytes cultured in suspension, by thymic as well as splenic APCs. Thus the recognition of antigen by CD4⁺CD8⁺ thymocytes may lead to deletion, suggesting that this is the central mechanism of tolerance induction, which is not limited by the antigen-presenting ability of the thymic stroma.

When thymocytes are cultured in single-cell suspension, a distinct population of cells appears which express reduced levels of CD4 and CD8 molecules. This population represents immature CD4⁺CD8⁺ thymocytes 'spontaneously' undergoing programmed cell death (apoptosis), which can be accelerated by known inducers such as ionomycin (Fig. 1 and ref. 11). To find out if this observation could be used to study the mechanism of antigen-specific induction of apoptosis in immature T cells, we used thymocytes from $\alpha\beta$ T-cell receptor (TCR) transgenic mice expressing, on more than 90% of CD4⁺CD8⁺ thymocytes, a TCR specific for the male (H-Y) antigen presented by class I major histocompatibility complex (MHC) molecules (H-2D^b)³.

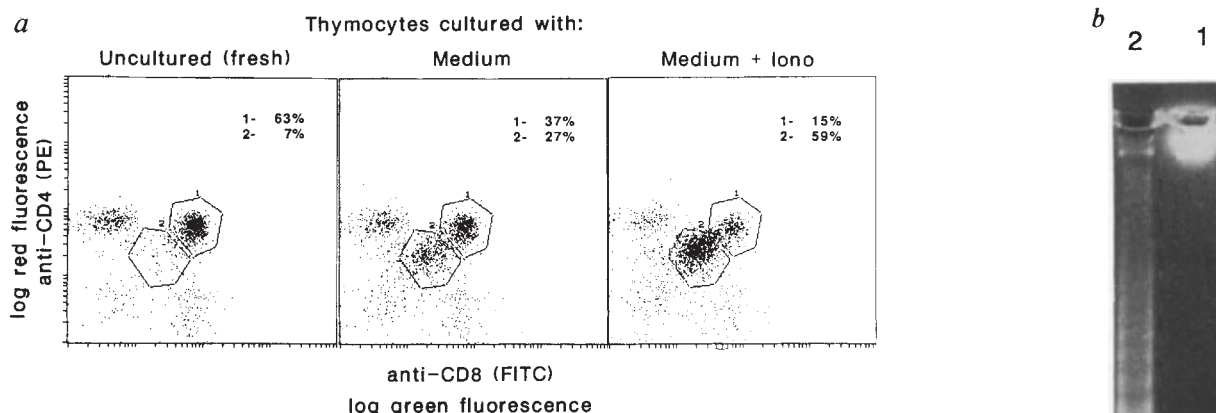


FIG. 1 Identification of CD4⁺CD8⁺ thymocytes undergoing apoptosis in suspension culture. **METHODS.** *a*, Thymocytes ($2 \times 10^6 \text{ ml}^{-1}$) from normal B6 mice were cultured for 24 h (37°C, 5% CO₂) in 24-well Costar tissue culture plates, in Iscove's modified Dulbecco's medium supplemented with $2 \times 10^{-5} \text{ M}$ 2-mercaptoethanol, penicillin (100 U ml^{-1}), streptomycin ($100 \mu\text{g ml}^{-1}$) and 10% FCS without or with ionomycin ($5 \mu\text{g ml}^{-1}$). The surface expression of CD4 and CD8 molecules on cell populations (from which only cell debris but not dead cells were excluded by forward and side-scatter gating) was analysed before and after *in vitro* culture by direct staining with the mixture of phycoerythrin (PE)-labelled anti-CD4 and FITC-labelled anti-CD8 monoclonal antibodies (Becton and Dickinson) as previously described³. Two populations of CD4⁺CD8⁺ thymocytes expressing high (gate 1) and low (gate 2) levels of CD4/CD8 molecules are shown. *b*, CD4/CD8 'high' and 'low' populations were sorted out by fluorescence-activated cell sorting, their DNA was isolated and analysed by agarose gel electrophoresis as described¹⁸. Lanes contained DNA isolated from CD4/CD8 'high' cells (lane 1) and 'low' cells (lane 2). Note that DNA in lane 2 is degraded into oligonucleosomal fragments, which is the characteristic feature of apoptosis, whereas DNA in lane 1 is not.

Thymocytes from H-2^b transgenic females were cultured with thymic or splenic adherent cells from B6 (H-2^b) males or females for different times, then collected and analysed for surface expression of CD4 and CD8 molecules. In control cultures, female transgenic thymocytes were cultured with male adherent cells from BALB/c (H-2^d) or AKR (H-2^k) mice and thymocytes from nontransgenic B6 female mice were cultured with male or female adherent cells (Fig. 2). During 24–48 h of culture, deletion of CD4⁺8⁺ thymocytes occurred only in the presence of cells bearing H-Y antigen with the appropriate (H-2^b) MHC molecules. During the first 12 h of coculture of transgenic thymocytes with H-2^b male APCs, more than 50% of CD4⁺8⁺ thymocytes underwent apoptosis as shown by decreased expression of CD4 and CD8 molecules; the proportion of which decreased only slightly in that time. After 48 h very few CD4⁺8⁺ thymocytes expressed normal levels of CD4 and CD8 molecules and the proportion of all CD4⁺8⁺ cells was much reduced. That apoptotic cells did not accumulate in culture later on, is probably due to their removal by

macrophages in the APC preparations as well as to desintegration of cells.

These changes are observed only with APCs expressing the appropriate antigen. Other APCs that express either the wrong MHC antigen or lack the male antigen do not seem to induce apoptosis in CD4⁺8⁺ thymocytes, as there was only a moderate decrease of the proportion of CD4⁺8⁺ cells expressing normal levels of CD4 and CD8 molecules; and the proportion of cells with reduced expression of CD4 and CD8 molecules did not significantly differ from the proportion of cells undergoing 'spontaneous' apoptosis at the beginning of culture. Similar results were obtained when transgenic thymocytes were cocultured with thymic accessory cells (data not shown, but see below). These experiments did not rule out the possibility that the deletion of CD4⁺8⁺ thymocytes is the consequence of antigen recognition by mature thymocytes present in suspension, which could release nonspecific factors toxic for immature thymocytes. This possibility was suggested by our observation that during ontogeny, deletion of CD4⁺8⁺ thymocytes in transgenic males

Time in culture (h)

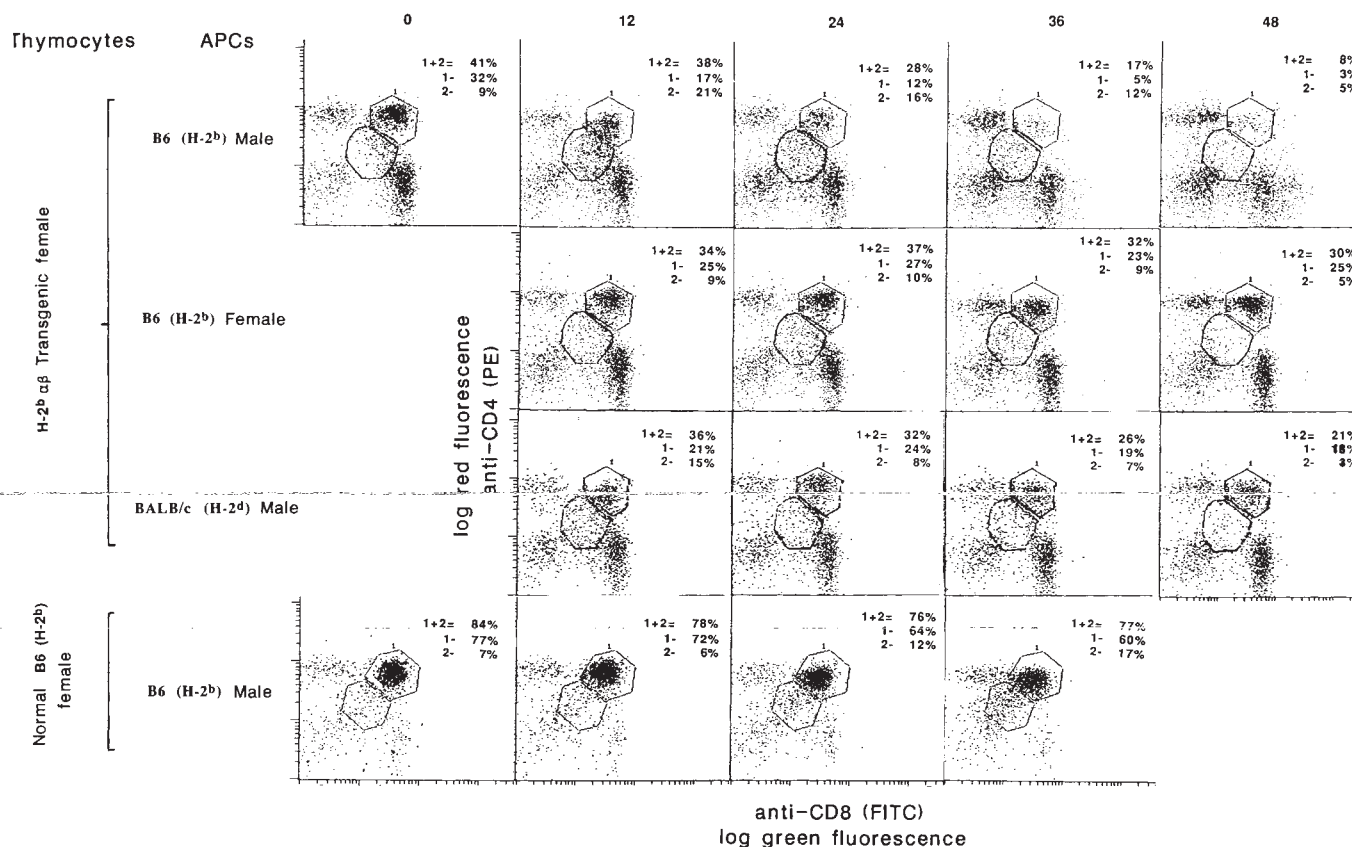


FIG. 2 *In vitro* antigen-specific induction of apoptosis of CD4⁺8⁺ thymocytes expressing transgenic TCR specific for male (H-Y) antigen in the context of H-2^b molecules. Thymocytes from H-2^b transgenic or normal female mice were cultured for the indicated period of time on the monolayers of adherent APCs, prepared from spleens of male or female mice of different H-2 haplotypes. Thymocytes were collected and analysed for expression of CD4/CD8 molecules. Similar results were obtained when thymic APCs were used instead of splenic APCs. There was no marked clonal deletion when APCs from male AKR (H-2^k) mice were cocultured with transgenic female thymocytes (not shown). In different experiments, in all control groups, the total numbers of thymocytes recovered after 48 h varied from 50–70% of the input with 5–15% fluctuations between them. The recovery of female transgenic thymocytes cocultured with B6 male APCs was 30–50% lower than in control groups.

METHODS. Monolayers of adherent thymic and splenic APCs were prepared as follows. Removed organs were placed in cold IMDM, cut into small (~1 mm²) fragments and washed free of the released cells by several rounds of resuspending, unit gravity sedimentation, and discarding cells

floating in the supernatant. The remaining tissue fragments were transferred into 16-mm wells of 24-well Costar tissue culture plates (10–20 fragments per well) and cultured in IMDM supplemented with 20% FCS (37 °C, 5% CO₂). During the first week, cultures were fed with fresh medium every 2 days by exchanging 50% of medium and then every second or third day washed to remove nonadherent cells. Thymocytes (2 × 10⁶ ml⁻¹) were added to these primary explants of adherent cell cultures when they reached ~75% confluency (one to three weeks after initiation of cultures). Similar results were obtained with freshly isolated splenic or thymic adherent APCs prepared by 2-h incubation (37 °C, 5% CO₂) of cell suspensions on Petri dishes followed by washing out of nonadherent cells and transfer of counted numbers of adherent cells (2 × 10⁶ per well) which were collected by incubation with trypsin-EDTA solution (Gibco). The CD4/CD8 phenotype of cultured thymocytes was analysed as previously described³. The preparations of adherent cells from spleen and thymus contained 40% and 20–30% of Mac1⁺ cells, respectively, less than 5% of CD4⁺, less than 5% of CD8⁺ and >95% class I MHC⁺ cells (data not shown).

was undetectable before day 18 of gestation¹², coinciding with the appearance of more mature subpopulations of thymocytes¹³.

To test whether apoptosis of CD4⁺8⁺ thymocytes resulted from a direct interaction with APCs, we separated CD4⁺8⁺ thymocytes from other thymocyte subpopulations by cell sorting (purity > 99%) and cocultured them with thymic or splenic APCs from B6 males or females. The removal of the mature thymocyte

populations did not affect the ability of male thymic APCs to induce antigen-specific apoptosis of CD4⁺8⁺ thymocytes expressing the transgenic TCR (Fig. 3). Unlike previous experiments, which were compatible with tolerance being due to an arrest of development^{3-6,14}, this result indicates that CD4⁺8⁺ thymocytes can be the target of antigen-specific deletion.

The ability of thymic as well as extrathymic APCs to induce

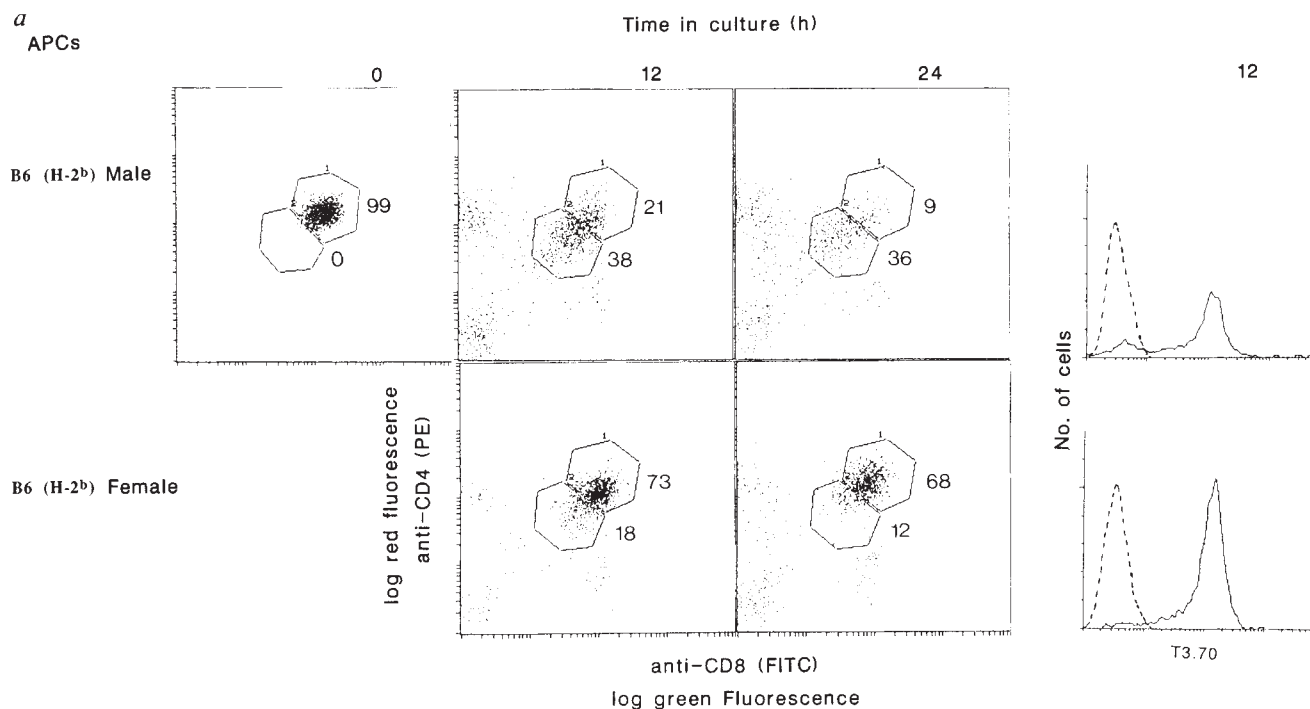
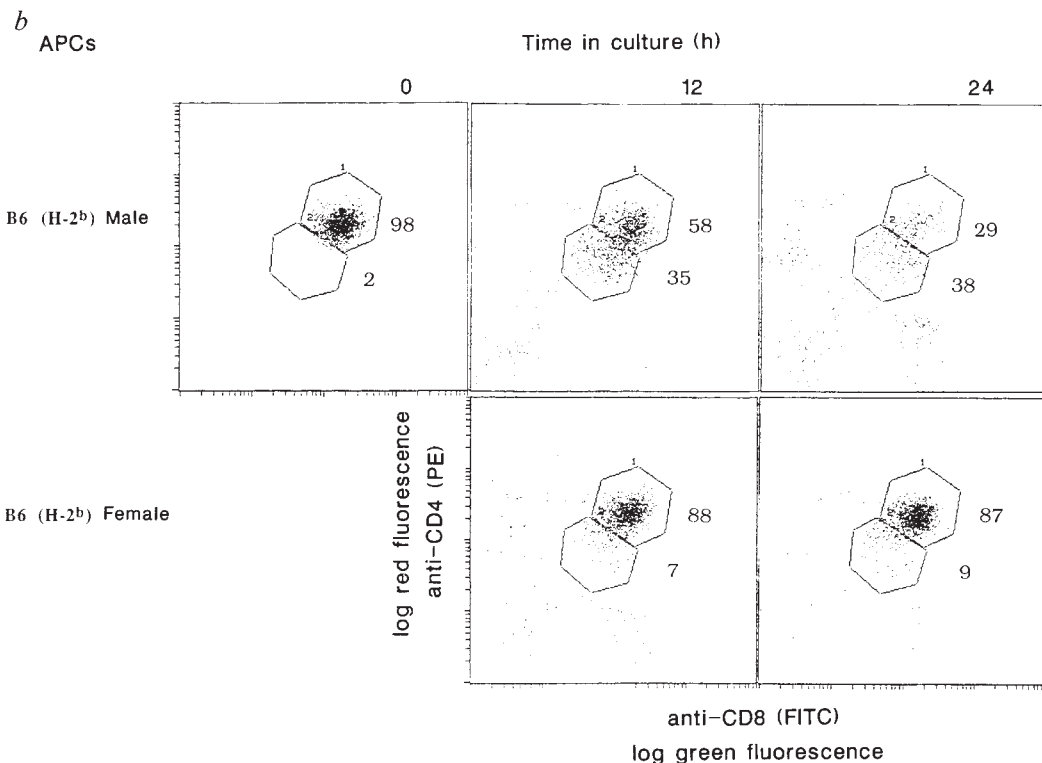


FIG. 3 Deletion by apoptosis of male antigen-specific CD4⁺8⁺ thymocytes results from direct antigen-specific interaction with APCs. The CD4⁺8⁺ thymocytes purified by cell sorting (>99% pure) were cultured on monolayers of thymic (a) or splenic (b) APCs and analysed for surface expression levels of CD4, CD8 and transgenic TCR molecules. Numbers indicate the proportions of viable CD4^{high}8^{high} (gate 1) and 'detectable' apoptotic CD4^{low}8^{low} (gate 2) cells.

METHODS. For detection of transgenic TCR expression, indirect staining with T3.70 monoclonal antibody (solid line), specific for transgenic alpha chain, was performed as previously described¹¹. Control staining with second-step reagent (FITC-labelled goat anti-mouse Ig) is indicated by broken line. The recovery of CD4⁺8⁺ thymocytes cultured on female or male APCs were

70% and 35% after 12 h and 45% and 10% after 24 h, respectively (a) and 80% and 50% after 12 h and 65% and 20% after 24 h, respectively (b). The CD4⁺8⁺, CD4⁺8⁺ and CD4⁺8⁺ cells, which can be seen on the dot plots, represent contaminating, nontransgenic lymphocytes surviving the



procedure of preparation of APCs. That they are of nontransgenic origin is indicated by the fact that the expression level of the transgenic TCR (T3.70) seen in a shows normal distribution, characteristic for the majority of CD4⁺8⁺ thymocytes¹².

antigen-specific deletion of CD4⁺8⁺ thymocytes indicates that it is not a unique property of the thymic microenvironment, but is the property of a certain developmental stage of T cells which makes it sensitive to deletion. This agrees with the recent study in thymic organ cultures from normal mice, in which the ability of peripheral dendritic cells to induce unresponsiveness in developing T cells by an unknown mechanism was shown¹⁵. Thus, antigens carried on APCs from the periphery into the thymus may induce central tolerance. The demonstration of antigen-specific deletion of CD4⁺8⁺ thymocytes in suspension culture offers new possibilities in the study of the selection mechanisms operating at this critical stage of T-cell development¹⁶. In particular, the role of various T-cell receptors and their ligands (for example, TCR, CD4, CD8, antigenic peptides¹⁷ and MHC molecules), as well as the role of different stromal cells, in determining whether deletion (apoptosis) or positive selection (inhibition of apoptosis) will result from interaction of immature thymocytes with thymic microenvironment, may be directly approached in a defined *in vitro* system. □

Received 8 November 1990; accepted 11 March 1991.

- Kappler, J. W., Roehm, N. & Marrack, P. C. *Cell* **49**, 273–280 (1987).
- MacDonald, H. R. *et al. Nature* **332**, 40–45 (1988).
- Kisielow, P., Blüthmann, H., Staerz, U., Steinmetz, M. & von Boehmer, H. *Nature* **333**, 742–746 (1988).
- Sha, W. C. *et al. Nature* **335**, 271–274 (1988).
- Fowlkes, B. J., Schwartz, R. H. & Pardoll, D. M. *Nature* **334**, 620–623 (1988).
- MacDonald, H. R., Hengartner, H. & Pedrazzini, M. *Nature* **335**, 174–176 (1988).
- Jenkinson, E. J. *et al. Eur. J. Immunol.* **19**, 2175–2177 (1989).
- von Boehmer, H. *A. Rev. Immunol.* **8**, 531–556 (1990).
- MacDonald, H. R. & Lees, R. K. *Nature* **343**, 642–644 (1990).
- Longo, D. L. & Schwartz, R. H. *Nature* **287**, 44–46 (1980).
- Swat, W., Ignatowicz, L. & Kisielow, P. *J. Immunol. Meth.* **137**, 79–97 (1991).
- Teh, H. S. *et al. Dev. Immunol.* **1**, 1–10 (1990).
- Ceredig, R., Dialynas, D. P., Fitch, F. W. & MacDonald, H. R. *J. exp. Med.* **158**, 1654–1660 (1983).
- White, J. *et al. Cell* **56**, 27–34 (1989).
- Matzinger, P. & Gunder, S. *Nature* **338**, 74–76 (1989).
- von Boehmer, H. & Kisielow, P. *Science* **248**, 1369–1373 (1990).
- Rotzschke, O. *et al. Science* **249**, 283–287 (1990).
- Smith, C. A. *et al. Nature* **337**, 181–183 (1989).

ACKNOWLEDGEMENTS. We thank P. Kusnierczyk for discussion, E. Palmer and W. Haas for reading the manuscript, E. Ziolo, J. Czech and K. Hafen for technical assistance. This work was supported by the Polish Academy of Sciences. The Basel Institute for Immunology was founded and is supported by F. Hoffmann-La Roche Ltd.

Parental imprinting of the mouse *H19* gene

Marisa S. Bartolomei, Sharon Zemel & Shirley M. Tilghman

Howard Hughes Medical Institute and Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544, USA

THE mouse *H19* gene encodes one of the most abundant RNAs in the developing mouse embryo¹. It is expressed at the blastocyst stage of development, and accumulates to high levels in tissues of endodermal and mesodermal origin (H. Kim, unpublished result). After birth the gene is repressed in all tissues except skeletal muscle. It lacks a common open reading frame in the 2.5-kilobase RNA, but has considerable nucleotide sequence similarity between the genes of rodents and humans^{2,3}. Expression of the gene in transgenic mice results in late prenatal lethality, suggesting that the dosage of its gene product is strictly controlled⁴. The *H19* gene maps to the distal segment of mouse chromosome 7, in a region that is parentally imprinted⁵, a process by which genes are differentially expressed on the maternal and paternal chromosomes. We have now used an RNase protection assay that can distinguish between *H19* alleles in four subspecies of *Mus*, to demonstrate that the *H19* gene is parentally imprinted, with the active copy derived from the mother. This assay will be of general use in assaying allele-specific gene expression.

To determine whether a gene is imprinted, one must be able

to distinguish expression from the maternal and paternal alleles. We used an RNase protection assay that is very sensitive to base substitutions and discontinuities in RNA sequences⁶. Hybrid F₁ mice generated from an interspecies cross between *Mus musculus domesticus* and *Mus spretus* were assayed for RNA levels transcribed from each *H19* allele. These two species diverged in evolution between 3 to 6 million years ago⁷, and exhibit significant nucleotide polymorphisms, a property that has been especially useful in genetic mapping strategies⁸. For the *H19* gene, the fifth exon is especially divergent in sequence between humans and mice³, and provides a likely candidate for harbouring an RNA polymorphism. An antisense RNA probe that included the 3' end of exon 3, all of exon 4 and the 5' part of exon 5 was derived from genomic DNA of the *M. domesticus* strain BALB/cJ (Fig. 1a). After hybridization to total liver RNA isolated from *M. domesticus* and *M. spretus* neonates, the RNase-resistant products were analysed by gel electrophoresis. *M. domesticus* RNA protected the expected full-length fragments corresponding to exons 3, 4 and 5. *M. spretus* RNA also fully protected exons 3 and 4 (date not shown), but a slightly

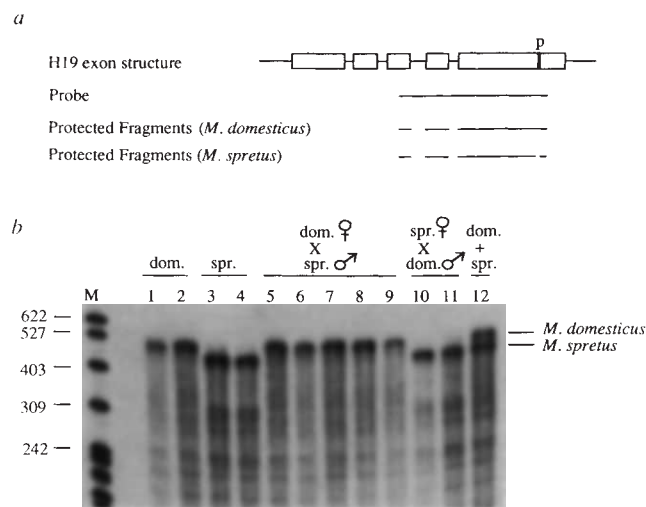


FIG. 1 RNase protection assay of liver RNA from *M. domesticus*, *M. spretus* and hybrid animals. *a*, The top line shows the exon structure of the mouse *H19* gene, drawn in a 5' to 3' orientation. Open boxes indicate exons and the lines correspond to introns and nontranscribed regions. The filled box in exon 5 and the corresponding 'p' above the box marks one possible site of the RNA polymorphism that exists between *M. domesticus* (dom.) and *M. spretus* (spr.). The second line shows a *Bam*HI–*Stu*I 754-base pair (bp) genomic DNA fragment that was used as the RNase protection probe. The probe fragments that are protected from RNase digestion following hybridization with *M. domesticus* or *M. spretus* *H19* RNA are drawn in the third and fourth lines, respectively. *b*, Autoradiogram of RNase protection assay products of 2 μg total liver RNA samples from neonatal mice. The F₁ progeny of *M. domesticus* females mated to *M. spretus* males (lanes 5–9) and of *M. spretus* females mated to *M. domesticus* males (lanes 10 and 11), as well as the parental lines *M. domesticus* (lanes 1 and 2) and *M. spretus* (lanes 3 and 4), are as indicated. Only the allele-specific protected fragment from exon 5 is included. Marker lane (M) contains radioactively labelled DNA fragments of *Msp*I-digested pBR322, with relative sizes indicated in base pairs. Lane 12 shows an assay control using 1 μg *M. domesticus* RNA and 1 μg *M. spretus* RNA. As both protected fragments display comparable intensities, the probe is equally capable of detecting both types of RNA. METHODS. The RNase protection was performed as described⁶ with the following exceptions. The *Bam*HI–*Stu*I fragment of the *H19* gene was cloned into the *Bam*HI–*Hinc*II sites of pBluescript II KS (Stratagene). The vector was linearized with *Xba*I, purified by agarose gel electrophoresis and used as a template in an *in vitro* transcription reaction using [³²P]CTP and T3 polymerase (Promega). Total RNA was prepared from day 2–5 neonatal animals by the lithium chloride/urea method¹⁹ and hybridized to 1 × 10⁵ c.p.m. of radiolabelled probe overnight at 65°C. After RNase digestion at 32°C for 1 h, samples were analysed by electrophoresis on a 6.6% acrylamide, 7 M urea gel, and exposed to Kodak XAR-5 X-ray film.

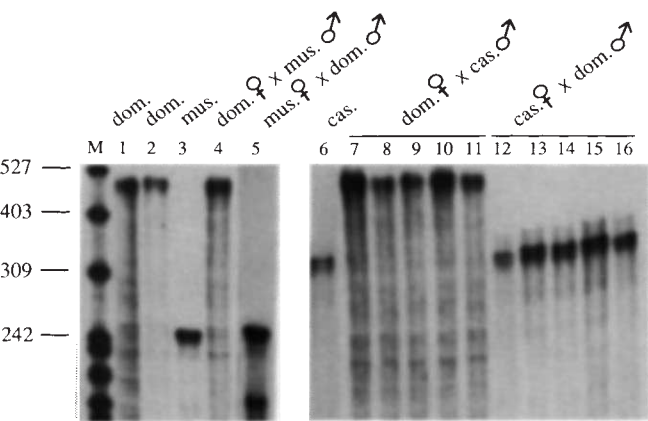


FIG. 2 RNase protection assay of skeletal muscle RNA from the progeny of *M. domesticus* crossed to either *M. castaneus* (cas.) or *M. musculus* (mus.). Total RNAs were isolated from skeletal muscle of 4-day-old *M. domesticus* (BTBR strain, 2 µg; lane 1), 3-month-old *M. domesticus* (CBA/NJX10/X10 strain, 10 µg; lane 2), 6-week-old *M. musculus* (SKIVE strain, 10 µg; lane 3), a 4-month-old F₁ mouse derived from a *M. domesticus* (CBA/NJX10/X10) female and *M. musculus* (Denmark) male cross (10 µg, lane 4) and a 6-week-old F₁ mouse from the reciprocal cross (10 µg, lane 5). Each RNA sample was subjected to the RNase protection assay, as described in the legend to Fig. 1. Total RNA derived from a *M. castaneus* neonate (1 µg, lane 6), individual 3-week-old progeny of *M. domesticus* (C57BL/6J strain) females crossed to *M. castaneus* males (5 µg, lanes 7–11) and progeny of *M. castaneus* females mated to *M. domesticus* males (lanes 12–16) were treated similarly. The protected fragments correspond to exon 5.

smaller exon 5 fragment was observed (Fig. 1b, compare lanes 1 and 2 to lanes 3 and 4).

When liver RNAs from neonatal progeny of *M. domesticus* females mated with *M. spretus* males were tested with the RNase protection assay, only the *H19* gene product characteristic of *M. domesticus* exon 5 was observed (Fig. 1b, lanes 5–9). Conversely, the reciprocal cross using *M. domesticus* males and *M. spretus* females resulted in progeny that expressed only the *M. spretus* *H19* allele (Fig. 1b, lanes 10 and 11). In both cases, only the allele inherited from the mother was expressed.

The exclusive expression of the *H19* maternal allele could be

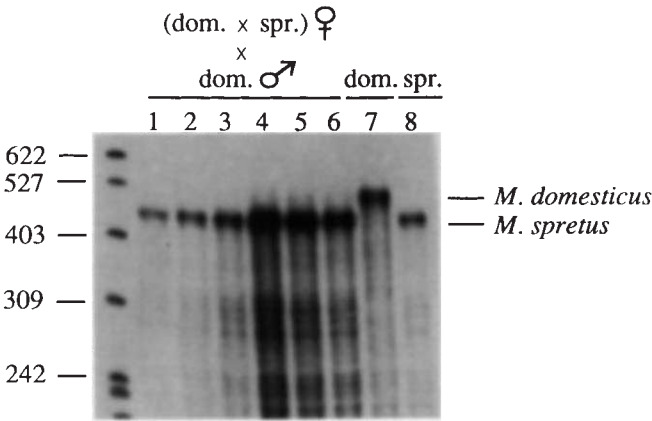


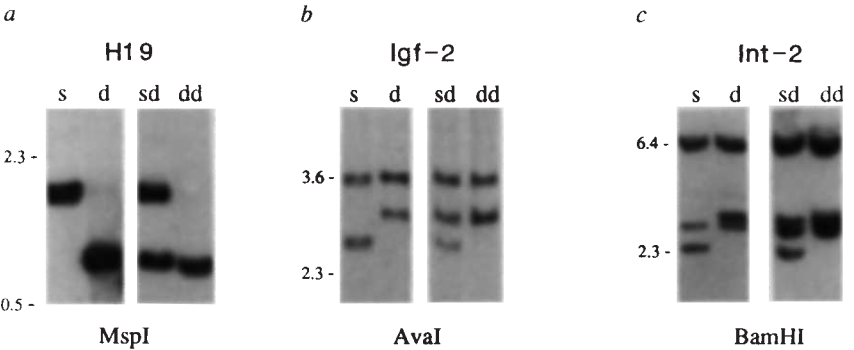
FIG. 3 Analysis of liver RNAs from *M. domesticus* × *M. spretus* backcross progeny. Female F₁ mice from a *M. domesticus* female × *M. spretus* male cross (Fig. 1) were mated with *M. domesticus* males and progeny were killed at different ages after birth. Total liver RNA isolated from progeny identified as being heterozygous for *H19* were assayed by RNase protection as described in Fig. 1: 1 day (1 µg, lanes 1 and 2), 4 days (2 µg, lane 3), and 1 week (5 µg, lanes 4–6). Liver RNA from homozygous *M. domesticus* and *M. spretus* was also assayed (2 µg, each, lanes 7 and 8, respectively). The protected fragments correspond to exon 5.

a unique property of the mouse strains used in this interspecific cross. We therefore analysed two additional crosses using different *Mus* species. Skeletal muscle RNAs from 2–3-month-old progeny from *M. domesticus* × *Mus musculus musculus* reciprocal crosses were assayed for the presence of *H19* RNA. The *M. musculus* RNA protects a fragment of about 240 bases, easily distinguished from the *M. domesticus* product (Fig. 2, lanes 1–3). Analysis of the hybrid progeny confirmed that only the maternally derived allele was expressed (Fig. 2, lanes 4 and 5).

Likewise, when skeletal muscle RNAs derived from F₁ progeny of *M. domesticus* × *Mus musculus castaneus* reciprocal crosses were analysed, the maternal allele was once again exclusively detected (Fig. 2, lanes 7–16). In addition to verifying the parental imprinting of the *H19* gene, these experiments illustrate that cells of both liver and skeletal muscle, derived from two

FIG. 4 Genetic mapping of the *H19* gene. a, b and c, Southern blots identifying RFLPs between *M. spretus* and *M. domesticus* using a 1.1-kb *Bam*HI *H19* genomic DNA fragment¹, a 550-bp rat *Igf-2* cDNA²⁰, and a 1.0-kb mouse *Int-2* cDNA²¹, respectively. In each panel the first two lanes show *M. spretus* (s) and *M. domesticus* (d; BALB/cJ strain) parental DNAs and the last two lanes show heterozygous (sd) and homozygous (dd) DNA from a (s × d) × d backcross. The restriction endonuclease used to digest the DNA is designated under each panel and relative size standards (in kb) are shown to the left. d, Number of recombinants detected between the *Int-2* and *H19* or *Igf-2* genes. The genetic distance represents the maximum distance defined at the 95% confidence level, calculated using the formula $0.05 = (1 - p)^N$, where *p* is map distance in Morgans and *N* is number of animals tested.

METHODS. Genomic DNA (15 µg) was digested and separated on a 1.0% agarose gel. DNA was transferred to Genescreen (NEN) by capillary transfer in 0.2 N NaOH/0.6 N NaCl. The filters were neutralized in 1 M Tris-HCl (pH 7.5), hybridized overnight using the conditions described by Church and Gilbert²² and washed for 2 h at 65°C in 0.015 M NaCl-0.0015 M sodium citrate/0.1% SDS. The probes were prepared by the method of Feinberg and Vogelstein²³ using [³²P]dCTP.



	Recombination interval	Number of recombinants	Map distance (cM)
d	<i>H19</i> - <i>Int-2</i>	0/110	<2.7
	<i>Igf-2</i> - <i>Int-2</i>	0/109	<2.7

different germ layers, display similar imprinting patterns, as does the neonatal gut (data not shown).

If the *H19* gene is indeed parentally imprinted, the pattern of allele-specific expression will be reset in the germ line at each generation. To test this, female F_1 (*M. domesticus* $\times$ *M. spretus*) mice, expressing the *M. domesticus* *H19* allele (Fig. 1b, lanes 5–9), were backcrossed to *M. domesticus* males. The progeny that inherited the *M. spretus* *H19* allele from the mother were identified by restriction fragment length polymorphism analysis of genomic DNA (data not shown). Analysis by RNase protection of liver RNAs from the progeny demonstrated that the maternally derived *M. spretus* allele was exclusively expressed (Fig. 3, lanes 1–6). This result demonstrates that the formerly silent *M. spretus* allele inherited from the mother was reactivated during oogenesis. A total of 24 backcross progeny were screened, and all displayed the exclusive expression of the *M. spretus* maternal allele. From this we also conclude that the imprinting requires sequences that are linked to the *H19* gene itself. In addition, the segregation of unlinked modifiers of imprinting, which can occur for imprinted transgenes^{9,10}, was not detected.

Both the *H19* gene¹ and the insulin-like growth factor-II gene (*Igf-2*) (T. Glaser and D. Housman, personal communication) map to mouse chromosome 7. Recently DeChiara *et al.* deleted the *Igf-2* gene by homologous recombination in embryonic stem cells¹¹. Heterozygotes who had inherited this inactive *Igf-2* gene from the father were smaller than normal. When the inactive gene was inherited from the mother, however, the heterozygote progeny were of normal size¹². The authors conclude that only the paternal allele of *Igf-2* is active in most expressing tissues, with the exception of the choroid plexus and the leptomeninges, where both alleles are active.

We used an interspecific backcross between the *M. domesticus* BALB/cJ strain and *M. spretus* to determine the genetic linkage between *H19* and *Igf-2*. Restriction fragment length polymorphisms that distinguish the alleles of the two genes (Fig. 4a–c) were assayed in the genomic DNAs of 110 progeny of a (BALB/cJ $\times$ *M. spretus*) $\times$ BALB/cJ backcross. No recombinants were observed (Fig. 4d). To position the genes on the chromosome 7 map, they were mapped relative to *Int-2*, which encodes a member of the fibroblast growth factor gene family, and which has been mapped to the distal third of chromosome 7 (ref. 13). No recombinants between *Int-2*, *H19* and *Igf-2* were detected, indicating that these genes are tightly linked. *H19* and *Igf-2* have been mapped to band 11p15.5 in the human genome¹⁴. This syntenic conservation would tend to discount our failure to detect recombinants being the consequence of an inversion on distal chromosome 7 of *M. spretus* relative to *M. domesticus*¹⁵.

That the *H19* and *Igf-2* genes are tightly linked, yet imprinted in opposite directions, implies that the signals for parental imprinting are unlikely to act over large chromosomal regions, in contrast to X chromosome inactivation¹⁶. Indeed, Barlow *et al.* showed that the *Igf-2r* gene, encoding the receptor for insulin-like growth factor-II, is expressed only from the maternally inherited chromosome 17 (ref. 17). But three other genes within a region of about 1.1 megabases containing *Igf-2r* are not subject to imprinting.

That the *H19* gene undergoes parental imprinting was suggested by two lines of evidence. First, introduction of additional copies of the *H19* gene in transgenic mice leads to late prenatal lethality, between 14 and 16 days of gestation⁴. Second, *H19* maps to a region which is parentally imprinted⁵. Using balanced translocations, Searle and Beechey demonstrated that a maternal duplication/paternal deficiency of distal chromosome 7 results in late prenatal lethality, whereas paternal duplication/maternal deficiency leads to early embryonic lethality¹⁸. The remarkable similarity in the timing of the lethal effects of extra copies of the *H19* gene in the transgenic experiments and the maternal duplications of chromosome 7 suggest strongly that they share the same aetiology: one or more extra copies of an active *H19*

gene. Whether this is the true cause of the lethality remains to be determined. The absence of *Igf-2* gene function, which would be the case in mice carrying the maternal duplications/paternal deficiencies, results in smaller mice, and therefore it alone cannot explain the prenatal lethality¹².

Whether the early embryonic lethality observed with paternal duplications/maternal deficiencies can be explained by loss of *H19* gene function will require the generation of such mutations through gene replacement in embryonic stem cells. Nevertheless, the studies described here are consistent with an important role for the *H19* gene in embryonic development in the mouse. That role remains unclear, however, given the unusual nature of the gene product, which is found in a highly abundant cytoplasmic RNA complex³. That even one extra copy of the gene may have specific deleterious effects implies that its abundance is being carefully controlled. □

Received 7 March; accepted 20 March 1991.

1. Pachnis, V., Belayew, A. & Tilghman, S. M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5523–5527 (1984).
2. Pachnis, V., Brannan, C. I. & Tilghman, S. M. *EMBO J.* **7**, 673–681 (1988).
3. Brannan, C. I., Dues, F. J., Ingham, R. S. & Tilghman, S. M. *Mol. Cell Biol.* **10**, 228–236 (1990).
4. Brunkow, M. E. & Tilghman, S. M. *Genes Dev.* (in the press).
5. Searle, A. G. & Beechey, C. V. in *Aneuploidy* (eds Dellarco, V. L., Voytek, P. E. & Hollaender, A.) 363–376 (Plenum, New York/London, 1985).
6. Melton, D. A. *et al.* *Nucleic Acids Res.* **12**, 7035–7056 (1984).
7. Bonhomme, F. *et al.* *Biochem. Genet.* **22**, 275–303 (1984).
8. Robert, B. *et al.* *Nature* **314**, 181–183 (1985).
9. Sapienza, C., Paquette, J., Tran, T. H. & Peterson, A. *Development* **107**, 165–168 (1989).
10. Allen, N. D., Norris, M. L. & Surani, M. A. *Cell* **61**, 853–861 (1990).
11. DeChiara, T. M., Efstratiadis, A. & Robertson, E. J. *Nature* **345**, 78–80 (1990).
12. DeChiara, T. M., Robertson, E. J. & Efstratiadis, A. *Cell* **64**, 849–859 (1991).
13. Davisson, M. T., Roderick, T. H., Hillyard, A. L. & Doolittle, D. P. *Mouse Newslett.* **81**, 12–19 (1988).
14. Glaser, T., Housman, D., Lewis, W. H., Gerhard, D. & Jones, C. *Somatic cell. molec. Genet.* **15**, 477–501 (1989).
15. Hammer, M. F., Schimenti, J. & Silver, L. M. *Proc. natn. Acad. Sci. U.S.A.* **86**, 3261–3265 (1989).
16. Lyon, M. F. *Am. J. hum. Genet.* **42**, 8–16 (1988).
17. Barlow, D. P., Stoger, R., Herrmann, B. G., Saito, K. & Schweifer, N. *Nature* **349**, 84–87 (1991).
18. Searle, A. G. & Beechey, C. V. *Genet. Res.* **56**, 237–244 (1990).
19. Auffray, C. & Rougeon, F. *Eur. J. Biochem.* **107**, 303–314 (1980).
20. Soares, M. B. *et al.* *J. molec. Biol.* **192**, 737–752 (1986).
21. Wilkinson, D. G., Peters, G., Dickson, C. & McMahon, A. P. *EMBO J.* **7**, 691–695 (1988).
22. Church, G. M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1991–1995 (1984).
23. Feinberg, A. P. & Vogelstein, B. *Analyt. Biochem.* **132**, 6–13 (1983).

ACKNOWLEDGEMENTS. We thank V. Chapman and S. Camper for providing *M. musculus* and *M. castaneus*, A. Efstratiadis for the rat *Igf-2* probe, and A. McMahon for the mouse *Int-2* probe. This work was supported by a NRSA postdoctoral fellowship (M.S.B.) and the NIH (S.M.T.). S.M.T. is an Investigator of the Howard Hughes Medical Institute.

Uptake of *Pneumocystis carinii* mediated by the macrophage mannose receptor

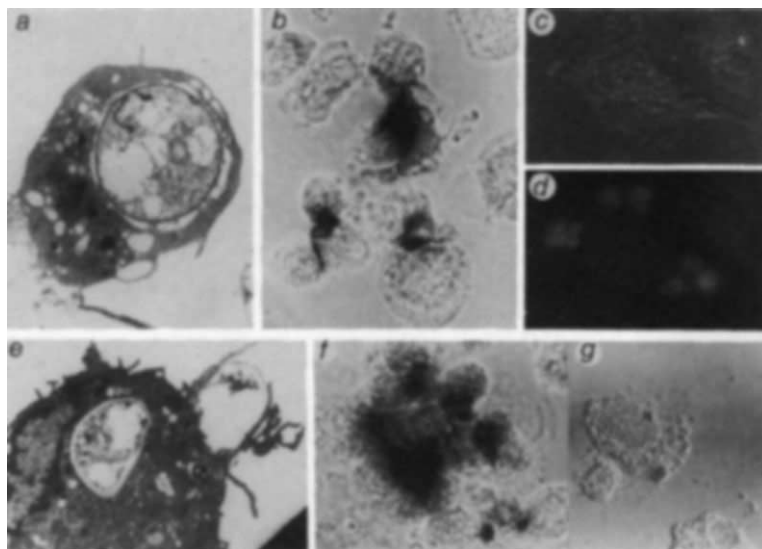
R. A. B. Ezekowitz*, D. J. Williams†, H. Koziel‡, M. Y. K. Armstrong§, A. Warner||, F. F. Richards† & R. M. Rose‡

* Division of Hematology/Oncology, Children's Hospital and The Dana Farber Cancer Institute, Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115, USA
 † MacArthur Center for Molecular Parasitology and Pulmonary and Critical Care Section, Department of Internal Medicine and § Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut 06510, USA
 ‡ Division of Pulmonary and Critical Care Medicine, New England Deaconess Hospital, Harvard Medical School, Boston, Massachusetts 02215, USA
 || Department of Respiratory Biology, Harvard School of Public Health, Boston, Massachusetts 02115, USA

HUMAN exposure to *Pneumocystis carinii* is common^{1,2} but, in the absence of acquired³ or genetic⁴ dysfunction of either cellular or humoral immunity, exposure rarely leads to illness. Although alveolar macrophages can degrade *P. carinii*^{5,6}, macrophage receptors involved in *P. carinii* recognition have not been clearly defined.

FIG. 1 *a*, An electron micrograph of rat alveolar macrophage that has ingested a typical thick-walled cyst form of *P. carinii*. *b*, *f*, Reduction of nitroblue tetrazolium, a histochemical dye that measures superoxide release, is triggered around labelled (*b*) and unlabelled (*f*) *P. carinii* after incubation with human alveolar macrophages. Organisms in a ratio of 10 organisms to one macrophage were incubated for 30 min at 37 °C. In parallel experiments, quantitative superoxide release was measured by the cytochrome *c* reduction assay¹³ and this resulted in 6 nmol O₂ release by 8×10^5 cells per 60 min. *c*, Human alveolar macrophages bind and ingest *P. carinii* as shown by differential interference contrast microscopy. *d*, The same field as in *c* viewed by fluorescence microscopy demonstrates that the organisms are readily visible, greatly enhancing accurate single-cell quantitation. *e*, Electron micrograph of rat alveolar macrophage having ingested an unlabelled *P. carinii*. *g*, Human alveolar bind and ingest unlabelled *P. carinii* as viewed by difference interference microscopy, but the unlabelled organisms are difficult to visualize.

METHODS. *P. carinii* were derived from the lungs of male Sprague-Dawley rats (Hilltop Laboratory Animals, Pennsylvania). After isolation they were propagated and purified in short-term cell culture as previously described^{10,21}. *P. carinii* from cell culture supernatants were washed three times in PBS, resuspended to 1×10^7 per ml in PBS containing 0.1 mg ml⁻¹ FITC (Sigma) and incubated at 37 °C for 30 min. They were then washed three times in PBS (10 ml at 4 °C or 22 °C for 20 min, then spun down at 900g) and stored in PBS at 4 °C in the dark until use. To avoid



clumping, the organisms were not resuspended in complex media until just before use.

Characterization of a predominant surface glycoprotein of the high mannose type^{7,8} led us to investigate the role of the macrophage mannose receptor in this process. We report here that binding and uptake of cultured rat *P. carinii* by human and rat alveolar macrophages is reduced by 90% in the presence of competitive inhibitors of mannose receptor activity and by adherence of alveolar macrophages to mannan-coated surfaces. Further, only those COS cells transfected with the human macrophage mannose receptor complementary DNA that express surface mannose receptors bind and ingest *P. carinii*. These studies establish that the macrophage mannose receptor is sufficient for uptake of *P. carinii* and emphasize the role of the alveolar macrophage in first-line host defence against *P. carinii*.

Because of the small size and refractility of unstained *P. carinii* organisms, they are difficult to see by light microscopy⁹. We thus devised an assay using fluoresceinated *P. carinii*. As fluorescein may alter the net surface charge on the organisms, we first had to validate that these labelled organisms are an effective substrate for phagocytosis. Rat and human alveolar macrophages expressing 5×10^5 per cell (ref. 10) were isolated by bronchoalveolar lavage and plated on glass coverslips. Cell monolayers were then incubated with *P. carinii* which had been propagated in short-term culture⁹. Phagocytosis of unlabelled *P. carinii* and organisms that had been labelled using the method described in the legend to Fig. 1 were compared. Figure 1*a*, *c*, and *d* shows that labelled organisms were taken up by alveolar macrophages and are indistinguishable from the unlabelled organisms (Fig. 1*e* and *g*). Labelling the organisms helped single-cell quantitation by allowing both fluorescence microscopy and differential interference contrast microscopy (Fig. 1*c* and *d*) to be used to distinguish bound versus ingested particles. The labelled organisms, like untreated organisms, bound to macrophages and some were also ingested (Fig. 1*a*, *c*, *d*, labelled; Fig. 1*e*, *g*, unlabelled). Studies with human alveolar macrophages in which 200 cells on three independent coverslips incubated with a ratio of 10:1 unlabelled or labelled organisms for 1 h gave a phagocytic index of 510 ± 60 and 540 ± 40 , respectively, with an average of five organisms per cell.

We next compared the ability of labelled and unlabelled *P. carinii* to trigger macrophage release of reactive oxygen intermediates. Labelled (Fig. 1*b*) and unlabelled *P. carinii* (Fig. 1*f*)

triggered the release of superoxides, as shown by the dye nitroblue tetrazolium, which formed insoluble blue deposits around the organisms. Both labelled and unlabelled organisms triggered the release of comparable amounts of superoxide after 1 h incubation at 37 °C (6 nmol and 6.5 nmol, respectively). Labelling did not seem to alter the ability of the organisms to serve as substrates for phagocytosis or to trigger a respiratory burst, and therefore provides a simple and qualitative method for studying the binding of *P. carinii* to the macrophage mannose receptor.

Rat alveolar macrophages were cultivated for 1 h at 37 °C in the presence of several concentrations of soluble competitive inhibitors of the mannose receptor. Dose-dependent inhibition

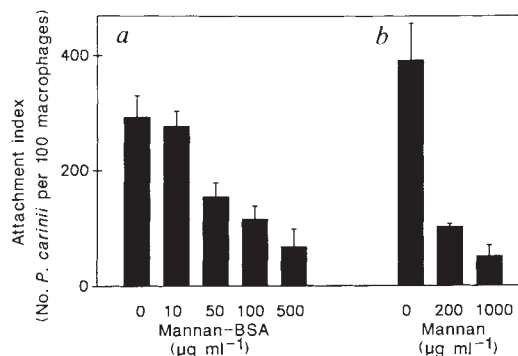
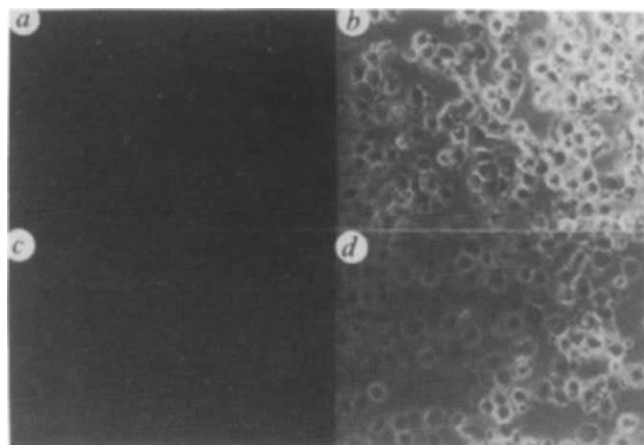


FIG. 2 Inhibition of binding of fluoresceinated *P. carinii* to rat alveolar macrophages by mannansylated BSA (*a*) and yeast mannan (*b*).

METHODS. Rat alveolar macrophages were isolated by standard lavage and plated on Lab-Tek 8-well slide chambers at 5×10^4 cells per well in RPMI plus 10% FCS. The cells were cultured for 24 h at 37 °C in 5% CO₂, washed in RPMI without serum and the indicated concentration of mannansylated BSA (E-Y Labs) or yeast mannan (Sigma) added for 20 min. Fluoresceinated *P. carinii* were then added at a final ratio of 20:1 (*P. carinii* to macrophages) and incubated for a further 60 min (final volume 0.2 ml) at 37 °C. The slides were then washed five times in PBS, fixed in 2% paraformaldehyde and the number of *P. carinii* associated per 100 macrophages counted in triplicate chambers by phase and fluorescent microscopy.

FIG. 3 Modulation of macrophage mannose receptor by mannan-coated coverslips, but not those coated with bovine serum albumin, reduced binding and uptake of fluoresceinated *P. carinii* by 90%. Substrata were prepared as described^{12,13}. Macrophages adhered to mannan surfaces showed 80% reduction in binding of ¹²⁵I-mannose-BSA. *a*, Fifty per cent of 2×10^5 human alveolar macrophages adhering to BSA-coated coverslips bound or ingested one or more fluoresceinated *P. carinii* (target to cell ratio 10:1; incubation time 40 min at 37 °C). *b*, Phase contrast micrograph of the same field. *c*, When they adhered to a mannan-coated substrate, less than 5% of human alveolar macrophages had bound or ingested one *P. carinii*. *d*, Phase contrast micrograph of the same field reveals a healthy cell monolayer. Cells adherent to mannan coverslips had 80% reduction in apical surface mannose receptors as detected by reduced binding of ¹²⁵I-mannose-BSA (not shown) and were able to ingest opsonized *Candida albicans*, indicating that the decreased binding and ingestion was selective. The methods used have been described elsewhere^{12,13}.

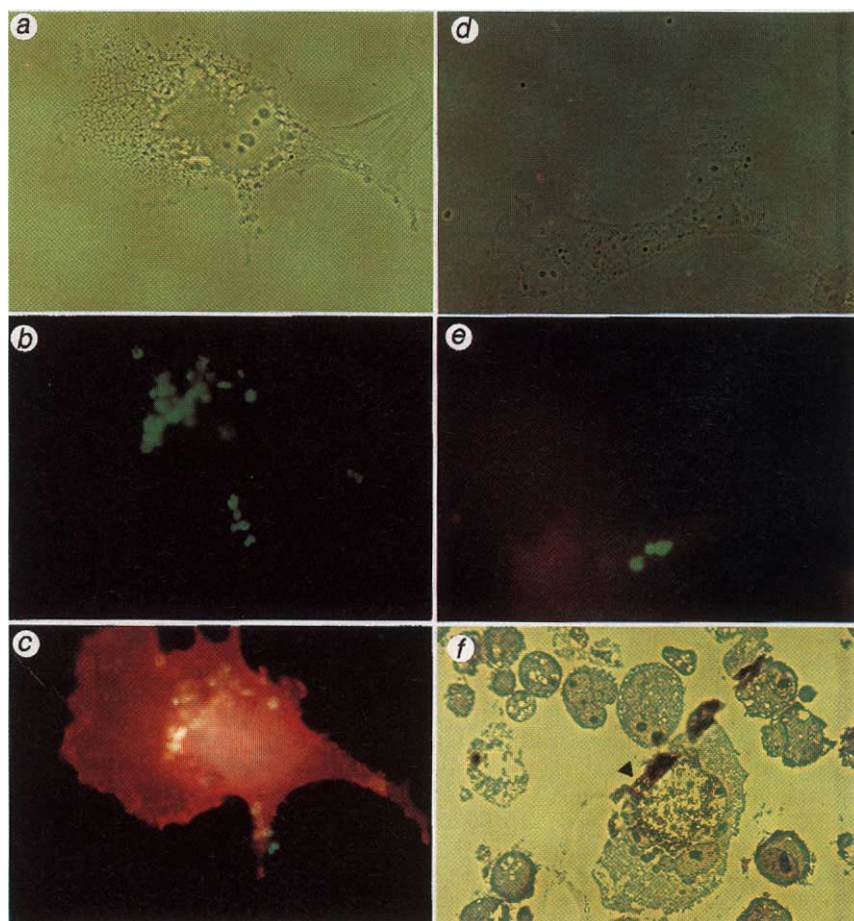


of *P. carinii* cell attachment was observed with both mannose-BSA, a neoglycoprotein (37 mol mannose: 1 mol BSA)¹¹, and mannose-rich yeast wall mannan extract (Fig. 2). There was 85% inhibition with 500 $\mu\text{g ml}^{-1}$ of human BSA and 90% inhibition with 1 mg ml^{-1} of mannan. In each experiment at least 300 cells were scored on triplicate slides. Mannose receptors were then selectively depleted from the macrophage plasma membrane by adherence to mannan-coated coverslips^{12,13}. Human alveolar macrophages adhering to mannose-coated coverslips had a 90% reduction in cell-associated *P. carinii* compared with macrophages plated on BSA substrata (Fig. 3*c* and *a*, respectively). Although macrophages adherent to mannan substrates

had greatly reduced numbers of bound and ingested labelled organisms, the cells had a typical macrophage appearance (Fig. 3*b* and *d*) and could ingest fluorescein isothiocyanate latex beads (not shown).

The recognition of other microorganisms by macrophages often involves cooperation between a number of cell-surface receptors on the macrophage surface¹⁴. A role for fibronectin in *P. carinii* cell adherence to epithelial cells and macrophages has been shown^{15,16}. To confirm that the mannose receptor was sufficient for *P. carinii* uptake, we examined COS-I cells that had been transfected with a full-length mannose receptor cDNA^{17,18}. This showed that a subpopulation of COS cells

FIG. 4 Mannose receptor transfected COS-I cells are able to bind and ingest *P. carinii*. *a*, Phase contrast micrograph of COS-I cell that had been transfected with a full-length macrophage mannose receptor cDNA^{17,22} and incubated with fluoresceinated *P. carinii* (cell to target ratio 15:1) for 30 min at 37 °C. The coverslips were then fixed with 3.5% formaldehyde and stained with a 1:100 dilution of a rabbit anti-mannose receptor antisera detected by an affinity-purified goat anti-rabbit rhodamine coupled immunoglobulin (Tago, California, USA). Mock transfected and untransfected COS cells failed to bind *P. carinii*. *b*, Photomicrograph of the same field as viewed under fluorescence microscopy. The *P. carinii* are more easily seen and appear green. *c*, Localization of mannose receptors and *P. carinii* as determined by the yellow staining of the majority of organisms. This results from the mixture of fluorescein and rhodamine. One organism remains green and is clearly extracellular. The cell shown is representative of all cells that had bound or ingested *P. carinii*. Of note is that only those cells that stained for the mannose receptor had associated with bound organisms. This constituted 20–30% of transfectants, which is the percentage of cells expressing mannose receptors as determined by FACS analysis. *d*, Phase contrast micrograph of mannose receptor-transfected COS-I cell that had been incubated with fluoresceinated *P. carinii* as described above, fixed and stained with normal rabbit antisera followed by a goat anti-rabbit rhodamine coupled immunoglobulin as a control for the specificity of the rhodamine coupled antibody. *e*, Dual fluorescence range of the field shown in *d* reveals green fluoresceinated organisms and a low background red stain. Exposure times were identical to those for *c*. *f*, Light micrograph of a 500-nm section showing unlabelled cysts within a COS cell transfected with mannose receptor cDNA. Sections were stained with toluidine blue which stains cysts and cell nuclei. Arrow indicates ingested *P. carinii*.



transfected with mannose receptor cDNA had specifically bound and ingested *P. carinii* (Fig. 4a, b). Double-fluorescence experiments revealed only those cells expressing surface mannose receptor had associated organisms. Colocalization of labelled organisms with mannose receptor (detected by rhodamine) membrane segments produced a yellow image (Fig. 4c). One organism still appeared green and is clearly not cell-associated. Another organism appeared half green (outside) and the other half is double-stained suggesting that the mannose receptor-containing membrane associated with the organism was in the process of enveloping the bound parasite (Fig. 4c). Controls for the specificity of the rhodamine staining (Fig. 4d) show phase contrast and double fluorescence of cells incubated with nonimmune rabbit sera followed by rhodamine-coupled second antibody (Fig. 4e). One aspect of the COS cells expressing the mannose receptor is that they were able to ingest the attached *P. carinii* (Fig. 4f). The ability of the mannose receptor to mediate phagocytosis in heterologous cells is consistent with

previous experiments 17.

In the immunocompetent host, the small numbers of organisms inhaled may be engaged by alveolar macrophage mannose receptors which result in release of reactive oxygen-intermediates and engulfment of the bound particle. But in immunocompromised states in man or experimental animal^{19,20}, an environment is created that favours the proliferation of *P. carinii* and the establishment of an extracellular infectious focus. Many factors may contribute to the failure of alveolar macrophages to clear the infection under these circumstances. It is possible that the mannose-rich surface proteins shed in *P. carinii* infection downregulate the expression and activity of mannose receptor, as has been shown with the mannose-rich yeast particle zymosan¹⁸. These abundant cell-surface proteins with high-mannose glycans may contribute to the pathobiology of *P. carinii* and may provide targets for therapeutic preparations of soluble forms of the mannose receptor or mannose-binding proteins. □

Received 20 November 1990; accepted 12 March 1991.

1. Pifer, L. L., Hughes, W. T., Stagno, S. & Woods, D. *Pediatrics* **61**, 35 (1978).
2. Meuwissen, J. H. E. Th., Tauber, I., Leeuwenberg, A. D. E. M., Becker, P. J. A. & Sieben, M. *J. infect. Dis.* **136**, 43 (1977).
3. Murray, J. F. et al. *Am. Rev. resp. Dis.* **135**, 504–510 (1987).
4. Masur, H. et al. *New Engl. J. Med.* **305**, 1431–1438 (1981).
5. von Behren, L. & Pesanti, E. A. *Rev. resp. Dis.* **118**, 1051–1059 (1978).
6. Masur, H. & Jones, T. C. *J. exp. Med.* **147**, 157–170 (1978).
7. Redding, J. A., Armstrong, M. Y. K., Ull, E. & Richards, F. F. *Infect. Immunity* **57**, 2149–2157 (1989).
8. Nakamura, Y., Tanabe, K. & Egoura, K. *J. Protozool.* **36**, 58s–60s (1989).
9. Armstrong, M. Y. K. & Richards, F. F. *J. Protozool.* **36**, 24s–27s (1989).
10. Ezekowitz, R. A. B. & Stahl, P. D. *J. Cell Sci.* **9**, 121–133 (1988).
11. Lee, Y. C., Stowell, C. P. & Krantz, M. J. *Biochemistry* **15**, 3956–3965 (1976).
12. Sung, S. J., Nelson, R. S. & Silverstein, S. C. *J. Cell Biol.* **96**, 160 (1983).

13. Ezekowitz, R. A. B., Sim, R. B., MacPherson, G. G. & Gordon, S. *J. clin. Invest.* **76**, 2368–2376 (1985).
14. Ezekowitz, R. A. B. in *Natural Immunity* (ed. Nelson, D. S.) 15–38 (Academic, Australia, 1989).
15. Pottratz, S. T. & Martin, W. J. *J. clin. Invest.* **85**, 351–356 (1990).
16. Pottratz, S. T. & Martin, W. J. *J. clin. Invest.* **86**, 1678–1683 (1990).
17. Ezekowitz, R. A. B., Sastry, K., Bailly, P. & Warner, A. *J. exp. Med.* **172**, 1785–1794 (1990).
18. Berton, G. & Gordon, S. *Immunology* **49**, 705 (1983).
19. Shellito, J. et al. *J. clin. Invest.* **85**, 1686–1693 (1990).
20. Harmsen, A. G. & Stankiewicz, M. *J. exp. Med.* **172**, 937–945 (1990).
21. Armstrong, M. Y. K. & Richards, F. F. *J. Protozool.* **35**, 24s–27s (1989).
22. Taylor, M. E., Conary, J. T., Lennartz, M. R., Stahl, P. D. & Drickamer, K. *J. biol. Chem.* **265**, 12156 (1990).

ACKNOWLEDGEMENTS. We thank F. McKoen, L. C. Benson, M. Perregaux, J. Fuglestad, Marsha Kartzman and Sue Brodha. This work is supported by NIH and the Cancer Research Institute (R.A.B.E.). D.J.W. is a Parker B. Francis Foundation Fellow.

Dependence of Ypt1 and Sec4 membrane attachment on Bet2

Guendalina Rossi, Yu Jiang, Anna P. Newman & Susan Ferro-Novick*

Department of Cell Biology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510, USA

MANY small GTP-binding proteins are synthesized as soluble proteins that are post-translationally modified as a prerequisite for membrane attachment¹. Ypt1 and Sec4 are homologous Ras-like GTP-binding proteins that have been proposed to regulate the specificity of vesicular traffic at different stages of the secretory pathway by cycling on and off membranes^{2–6}. Here we show that *BET2*, initially identified as a gene required for transport from endoplasmic reticulum to Golgi apparatus in yeast⁷, encodes a factor that is needed for the membrane attachment of Ypt1 and Sec4. DNA sequence analysis has revealed that Bet2 is homologous to Dpr1 (Ram1), an essential component of a protein prenyl-transferase that modifies Ras⁸, enabling it to attach to membranes^{9,10}. We propose that Bet2 modifies Ypt1 and Sec4 in an analogous manner.

We determined whether the *BET2* gene interacts with other genes whose products mediate secretion in yeast by crossing *bet2* to a representative of each of the complementation groups of secretory mutants that block vesicular transport from the endoplasmic reticulum to the Golgi complex (*bet1*, *sec12*, *sec13*, *sec16*, *sec17*, *sec18*, *sec20*, *sec21*, *sec22*, *sec23*), through the Golgi apparatus (*sec7*, *sec14*), and from the Golgi to the cell surface (*sec1*, *sec2*, *sec3*, *sec4*, *sec5*, *sec6*, *sec8*, *sec9*, *sec10*, *sec15*). We found that double mutant strains containing *bet2-1* in combination with *sec4-8* or with a mutation in genes (*sec2-41*, *sec5-24*, *sec8-9* and *sec15-1*) that strongly interact with *sec4-8* (ref. 5),

are inviable or nearly inviable at a temperature at which each single mutant grows well (25 °C, see Table 1). The same phenomenon was also observed when *bet2-1* was combined with *ypt1-2* (ref. 3), or *sec21-1*, a mutation in a gene that interacts genetically with *ypt1-2* (ref. 3). Therefore, in addition to interacting genetically with *YPT1*, *BET2* interacts with *SEC4* and with those genes previously shown to interact with either *YPT1* or *SEC4*. These findings could reflect a physical interaction between Bet2 and both Ypt1 and Sec4. Alternatively, Bet2 may perform a similar function on a pathway parallel to that of Ypt1 and Sec4.

One explanation for these genetic findings is that Bet2 is necessary to maintain the function of both Ypt1 and Sec4. As many GTP-binding proteins function when membrane-bound, they may be defective for function in the *bet2-1* mutant because they fail to attach to membranes. To test this hypothesis, the membrane association of Ypt1 and Sec4 was examined in wild-type and mutant cells after incubation at either 25 °C or 37 °C. At either temperature, the soluble pool of both proteins (Fig. 1a, lanes 5 and 2, b, lanes 7 and 3) was substantially larger in the mutant than in the wild type, and the fraction associated with membranes (Fig. 1a, lanes 6 and 3, b, lanes 8 and 4) was nearly absent or largely diminished (see legend to Fig. 1). For Ypt1, we also observed the presence of a degradation product in the soluble pool of wild type (Fig. 1a, lanes 1 and 2) which was reduced in quantity in the mutant (Fig. 1a, lanes 4 and 5). The failure of Ypt1 to attach to membranes was not an indirect consequence of blocking traffic from endoplasmic reticulum to Golgi or traffic within the Golgi apparatus, as its association with membranes was still seen in other secretory mutants that block transit at this stage of the pathway (not shown). These localization studies predict that, in the *bet2-1* mutant, loss of function will be more severe for Ypt1 than Sec4. The phenotypic analysis of this mutant, described below, shows this to be so. The Bet2 protein does not mediate the membrane attachment of all small GTP-binding proteins. Rather, it seems to function in conjunction with a particular class of proteins, as the localiz-

* To whom correspondence should be addressed.

ation of Ras2 (Fig. 1*b*, compare lanes 1, 3 and 4 with lanes 5, 7 and 8), as well as Ras1 (not shown), is unaltered in this mutant.

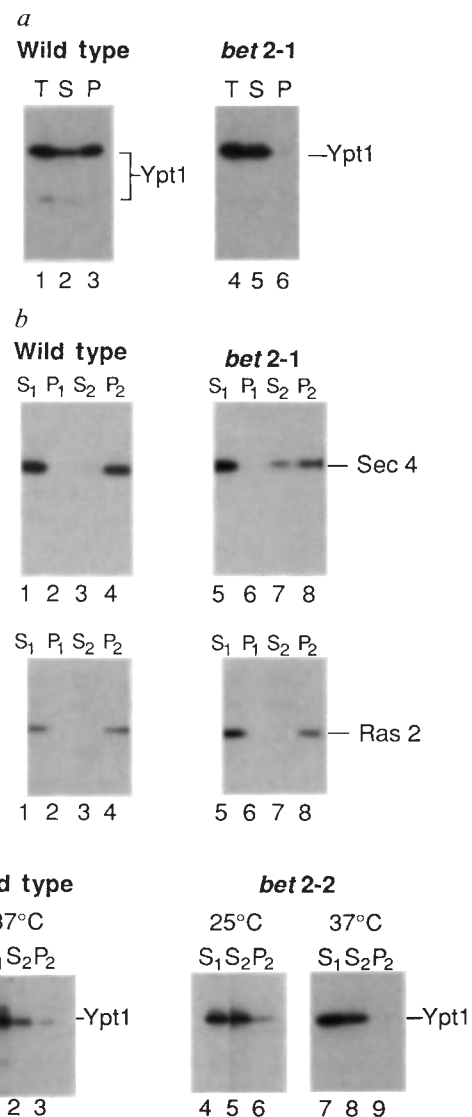
To demonstrate that the activity of Bet2 causes Ypt1 to attach to membranes, we examined the distribution of newly synthesized protein in a strain carrying a second mutant allele of this gene, *bet2-2* (previously identified as *orf2-1*)¹¹. Although we found that Ypt1 was attached to membranes in permissively grown *bet2-2* cells (Fig. 1*c*, compare lanes 4, 5 and 6 with 1, 2 and 3), it was completely soluble if the cells were incubated at 37 °C before fractionation (Fig. 1*c*, lanes 7, 8 and 9). This indicates that, in the *bet2-2* mutant, the attachment of Ypt1 to membranes is temperature-sensitive. In contrast, the membrane association of Ypt1 is defective at both 25 °C and 37 °C in the *bet2-1* strain. At 25 °C, the growth of *bet2-1* is not severely impaired. Death does, however, ensue when the cells are shifted to 37 °C. One explanation for this phenomenon is that the requirement for Ypt1 and Sec4 function is increased at 37 °C, exacerbating the constitutive defect of *bet2-1* at this raised temperature.

If, as we propose, the Ypt1 protein is defective for function in the *bet2* mutant, then this mutant should display the properties of a *ypt1* mutant. As for *ypt1-2* (ref. 3), *bet2-1* mutant fractions fail to support the late events that take place in an *in vitro* assay (not shown) that reconstitutes endoplasmic reticulum to Golgi and intra-Golgi transport^{12,13}. In addition, we compared the form of invertase secreted at 37 °C by *bet2-1* with that secreted by *ypt1-1* mutant cells (Fig. 2). In the *ypt1-1* mutant, the core glycosylated or endoplasmic reticulum form of invertase accumulates intracellularly at the restrictive temperature, whereas an underglycosylated form of this protein is secreted² (also see Fig. 2, lanes 6 and 5, compare lanes 2 and 1). The *bet2-1* mutant also has this phenotype (Fig. 2, lanes 4 and 3). Thus, the invertase that fails to accumulate in the endoplasmic reticulum lumen of *bet2-1*, does not collect in post-Golgi secretory vesicles as it does in *sec4*, but instead is secreted as an underglycosylated protein as in the *ypt1-1* mutant.

One aspect in which Ypt1 and Sec4 differ from yeast Ras is that they have a CC motif at their carboxy terminus rather than

FIG. 1 A defect in the Bet2 protein leads to an increase in the soluble pool of Ypt1 and Sec4 but not Ras. *a*, Lane 1, wild-type lysate (T); lane 2, wild-type soluble fraction (S); lane 3, wild-type insoluble fraction (P); lane 4, *bet2-1* mutant lysate (T); lane 5, *bet2-1* soluble fraction (S); lane 6, *bet2-1* insoluble fraction (P). *b*, Lane 1, supernatant of wild-type lysate subsequent to a spin at 450g (*S*₁); lane 2, pellet from the 450g spin (*P*₁); lane 3, wild-type soluble fraction (*S*₂); lane 4, wild-type insoluble fraction (*P*₂); lane 5, *S*₁ from *bet2-1*; lane 6, *P*₁ from *bet2-1*; lane 7, *S*₂ from *bet2-1*; lane 8, *P*₂ from *bet2-1*. *c*, Lane 1, *S*₁ from wild type (37 °C); lane 2, *S*₂ from wild type (37 °C); lane 3, *P*₂ from wild type (37 °C); lane 4, *S*₁ from *bet2-2* (25 °C); lane 5, *S*₂ from *bet2-2* (25 °C); lane 6, *P*₂ from *bet2-2* (25 °C); lane 7, *S*₁ from *bet2-2* (37 °C); lane 8, *S*₂ from *bet2-2* (37 °C); lane 9, *P*₂ from *bet2-2* (37 °C).

METHODS. *a*, Wild-type (SFNY26-6A; *MATα*, *his4-619*) and *bet2-1* (ANY119; *MATα*, *bet2-1*, *ura3-52*, *his4-619*) mutant cells were grown overnight at 25 °C in yeast peptone dextrose medium to an early exponential phase. One sample of cells (150A₅₉₉ units) was converted to spheroplasts during incubation for 1½ h at 25 °C in spheroplast medium⁷. A second was incubated at 37 °C for 1 h and then converted to spheroplasts during 1 h incubation at 37 °C. The spheroplasts were washed, lysed in 6 ml ice cold lysis buffer described before¹⁸, and the lysate (T) was homogenized with a Wheaton tissue grinder. Unbroken cells were removed during a 3min spin at 450g and the supernatant of this spin (*S*₁) was centrifuged at 200,000g for 1 h to generate a soluble fraction (S or *S*₂). The pellet (P or *P*₂) was resuspended in a volume of lysis buffer equal to the supernatant. Samples were electrophoresed and western blotted using anti-Ypt1 antibody. A degradation product of the Ypt1 protein (*M*_r 21,000), is seen on the blot shown in *a*. The Ypt1 protein has an *M*_r of 23,000. The blots from several experiments were measured and the percentages of the total (and standard deviations) are given in this legend. From these blots we determined that the soluble pool of the mutant (84.3 ± 1.15%) was substantially larger when compared to wild type (46.3 ± 6.8%)¹⁵, whereas the insoluble fraction was nearly absent (7.66 ± 2.5% in the mutant compared to 36.66 ± 11% for wild type). The results obtained were independent of the temperature of the incubation. *b*, Wild-type (SFNY 96; *MATα*, *ura3-52*, *pYcp50-ADH RAS2*) and *bet2-1* mutant cells (SFNY 98; *MATα*, *bet2-1*, *ura3-52*, *his4-619*, *Ycp50-ADH RAS2*), harbouring the *RAS2* gene on a *Ycp50-ADH* vector, were fractionated as described above. Samples were electrophoresed and western blotted using either anti-Sec4, or anti-Ras antibody, as described before^{18,22}. When the blots from several experiments were measured, the soluble pool of Sec4 in the mutant was found to be larger (48.4 ± 8.57%) when compared to wild type (17.2 ± 7.9%)¹⁸, whereas the insoluble fraction was greatly reduced (38.6 ± 8.23% compared to 79.2 ± 3.56% for wild type). The results obtained for the Sec4 protein were independent of the temperature at which the incubation was performed. *c*, To analyse the distribution of newly synthesized Ypt1 protein, wild-type (YF1593)¹¹ and *bet2-2* mutant cells (YF1594)¹¹ were grown overnight at 25 °C and radiolabelled at the permissive and restrictive temperatures. A portion of each culture (4A₅₉₉ units) was labelled with 250 μCi of trans [³⁵S]label at 25 °C (10 min); a second aliquot of cells (4A₅₉₉ units) was radiolabelled for 10 min at 37 °C, after incubation (15 min or 1 h) at this temperature. (The same results were obtained with preincubation at 37 °C for either 15 min or 1 h.) All samples were then fractionated, as described above, and Ypt1 was immunoprecipitated from each fraction⁷. The distribution of Ypt1 in wild type was identical when the cells were incubated at either 25 °C (not shown) or 37 °C (lanes 1, 2 and 3). The wild-type soluble pool was, however, found to be larger (~2/3 is soluble



and 1/3 is membrane bound) than reported in the legend to *a*. This variation may be a consequence of the strain background, because the wild-type strains used in the experiments shown in *a* and *c* were nonisogenic. The data in *c* is representative, as this experiment was repeated several times and the results were always identical. We have also examined the membrane association of newly synthesized Ypt1 in the *bet2-1* mutant (ANY 119) and SFNY 26-6A at 25 °C and 37 °C and found the distribution of this protein to be the same as reported in *a*.

TABLE 1 Tetrad analysis

	Number of tetrads								
	Four*	Three†			Two‡				
	4-0+§	3-1+	2-2+	3-0+	2-1+	1-2+	2-0+	1-1+	0-2+
<i>bet2-1</i> crossed with									
<i>sec2-41</i>	6			11			1	2	5
<i>sec4-8</i>	4			15		1			3
<i>sec5-24</i>	3			7				2	1
<i>sec8-9</i>	4	1		14		1			2
<i>sec15-1</i>	5			5					2
<i>sec21-1</i>				8					3

When *bet2* was crossed to the yeast secretory mutants listed above, either four, three or two of the four spores obtained were unimpaired for growth at 25 °C. The spores impaired for growth always contained both secretory mutations in the same haploid strain. This was revealed by analysis of the remaining spores in each tetrad. Haploid double mutant strains containing *bet2-1* in combination with either *sec4-8*, *sec5-24*, *sec8-9*, *sec15-1* or *sec21-1* were nearly invariable at 25 °C. Double mutants containing *bet2-1* in combination with *sec2-41* were inviable. Strains containing *bet2-1* in combination with either *sec1-1*, *sec6-4*, *sec7-1*, *sec12-4*, *sec13-1*, *sec14-3*, *sec16-2*, *sec17-1*, *sec18-1*, *sec22-3*, *sec23-1* or *bet1-1* were not impaired for growth; small growth defects were observed at 25 °C when *bet2-1* was combined with either *sec3-2*, *sec9-4*, *sec10-2*, *sec19-1* or *sec20-1* in the same haploid strain.

* All four spores exhibited unimpaired growth at 25 °C.
† Three of the four spores exhibited unimpaired growth at 25 °C.
‡ Two of the four spores exhibited unimpaired growth at 25 °C.
§ Designates the number of spores that grew (+) or did not grow (-) at 37 °C.

a CAAX box (C, Cys; A, aliphatic residue; X, any amino acid). The CAAX box signals a series of modifications that are required for membrane binding. These events include prenylation, proteolytic processing, and carboxymethylation¹⁴. Although the mechanism of membrane attachment of Ypt1 and Sec4 is unknown, the carboxy-terminal CC motif of these proteins may serve a similar function^{15,16}. As a first step towards determining the mechanism by which Bet2 is required for the membrane attachment of Ypt1 and Sec4, we have cloned and sequenced the gene that encodes this protein. DNA sequence analysis has revealed that the *BET2* gene encodes a hydrophilic protein of relative molecular mass ~36,500 (*M_r* 36.5K), which shares 56.6% similarity and 34.1% identity with Dpr1 (Ram1), an essential component of a prenyltransferase that modifies the carboxy terminus of Ras and the yeast mating pheromone α -factor⁸. A comparison of the Bet2 and Dpr1 (Ram1) proteins is shown in Fig. 3. *BET2* is also the same as *ORF2*, an unidentified open reading frame that encodes an essential protein of unknown function¹¹. The DNA sequences of these genes are identical with one exception which results in a conserved amino-acid change at position 22 (asparagine for Orf2 and lysine for

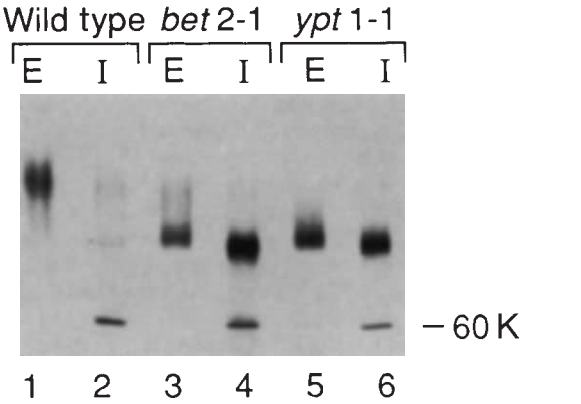


FIG. 2 The *bet2* mutant secretes an underglycosylated form of invertase. The following samples were immunoprecipitated with anti-invertase antibody and analysed on a 12.5% SDS polyacrylamide gel: lane 1, wild-type (SFNY 93; *MAT α* , *ura3-52*, *his4-619*, *pRB58*²³) external invertase (E); lane 2, wild-type internal invertase (I); lane 3, *bet2-1* (SFNY 92; *MAT α* , *bet2-1*, *ura3-52*, *his4-619*, *pRB58*) external invertase (E); lane 4, *bet2-1* internal invertase (I); lane 5, *ypt1-1* (SFNY 94; *MAT α* , *ypt1-1*, *ura3-52*, *pRB58*) external invertase (E); lane 6, *ypt1-1*, internal invertase (I). METHODS. Cells were grown at 25 °C, shifted to 37 °C for 30 min, and radiolabelled at this temperature (45 min). The invertase retained within the cells and secreted was immunoprecipitated, and electrophoresed as described before⁷. Two forms of invertase, a constitutively synthesized cytoplasmic form, and a secreted form, were observed. The cytoplasmic form, of *M_r* 60,000 (60 k), is seen in lanes 2, 4 and 6.

Bet2) of the protein. This exception may be a consequence of differences in strain backgrounds. *ORF2* and *DPRI* (*RAM1*) were previously reported to be homologous¹¹, but the significance of this homology has been unclear until now.

The mechanism by which Ypt1 and Sec4 associate with membranes has so far remained elusive. Although the presence of an isoprene group has not been identified on this class of proteins, our data implies that Bet2 is a component of a protein prenyltransferase that attaches an isoprenoid moiety to the C-terminus of Ypt1 and Sec4. Consistent with this hypothesis is the observation that the soluble pool of Sec4 is exaggerated in a mutant¹⁷ that blocks the synthesis of mevalonic acid (P. Novick, personal communication), a precursor of all isoprenoids. As it has been proposed that Ypt1 and Sec4 regulate vesicular traffic by cycling on and off membranes^{6,16,18}, the modification of these proteins may be subject to turnover. This would be unlike the case for Ras where the prenylation event is irreversible¹⁴. Alternatively, an isoprenoid group may be stably

Dpr1(Ram1)	61	LOSLEIYDDE..KNIEPALTKEFHMYLDVAFEISLPPOMTALDASQW	108
Bet2	1	MSGSLTLLEKHIRYIESLDTKKH.....FEVLTETHLRLNG.....	38
Dpr1(Ram1)	109	MLYIANSILKVMORDVSDDTKRKIVVKLFTISPSG...GPFGGGQGLS	155
Bet2	39	LYWGLTALCVLD...SPETVVKVIVSFVLSQWQYGAFAFPFRHDA	83
Dpr1(Ram1)	156	HLASTYAAINALSLCNDIDGCDRIDRKGIYQWLISLKEPNCGFKTCLF.	204
Bet2	84	HLTLTSAVQILATYDALD....VLGDKRVRLISFIRGNQLEDGSPFG	128
Dpr1(Ram1)	205	..VGEVDTRIGLYCALSIATLLNLTETLFTGVLYNLYKNCQNYEGFGS	252
Bet2	129	DRFGVDTRFYVYALSILGELTSEVVDPAVDVFLKCNFDDGGFGLCP	178
Dpr1(Ram1)	253	HVDEAHGGYTPCATASLAILRSMQINVEKLE...WSSARQLOEERGF	299
Bet2	179	N.AESHAQAFTCLGALAIANKLMDLSDQLEIGWVLCEROLPEG..GLN	226
Dpr1(Ram1)	300	GRSNKLVDCGYCFVWGGSAAILAFAFYGCNFKHALRDYIYCCQKEOP	349
Bet2	227	GRPSKLPDVCYSWVWLSLAIIGRLDW...INYEKLTEFLL.KCODEKKG	272
Dpr1(Ram1)	350	GLRDKPGAHSDFYHTNYCLGLAVAESSYSCTPNDSPHNIKCTPDLRIGS	399
Bet2	273	GIDRPNENEVDVHFVTFVGAAGLS.....LMGY	299
Dpr1(Ram1)	400	SKLTDVNPVYGLPIENVRKIIHY	422
Bet2	300	DNLVPIDPIYCMKSVTSKFKKY	322

FIG. 3 A comparison of the Bet2 and Dpr1 (Ram1) proteins. Single-letter amino-acid code used. *BET2* was cloned by complementation of the *bet2-1* growth defect, using a plasmid library containing inserts of genomic DNA inserted into the YCp50 vector²⁴. A complementing fragment (~2.0 kilobases), cloned into Ylp5, was shown to integrate at the *bet2* locus. To do this, we crossed a *MAT α* , *bet2-1*, *ura3-52* strain to a *MAT α* , *his4-619*, *ura3-52*, *BET2::URA3*, *BET2* strain and in 23 tetrads, the Ura⁺ and Ts⁺ phenotypes always cosegregated. A *HindIII* site, which were shown to be internal to the *BET2* gene by complementation studies, is contained within the open reading frame encoding the sequence shown in this figure. The 2.0-kb complementing fragment, inserted into pNRB113 (a 2 μ m plasmid vector), also complemented the growth and membrane localization defects of the *bet2-1* mutant. The amino-acid sequence of *BET2* (total sequence 325 amino acids) is compared to *DPRI* (*RAM1*)²⁵ using the BESTFIT program. Identity is indicated by a line and conserved changes are marked by two dots (two corresponding bases in their codons) or one dot (one corresponding base in their codons) between the sequences. The gaps are designated by dots within a sequence.

attached to Ypt1 and Sec4 and a second element could be involved in releasing these proteins from membranes.

Bet2 seems to act on a different family of proteins from Dpr1 (Ram1), as we have found that yeast Ras is membrane-bound (Fig. 1b) and α -factor is biologically active in the *bet2* mutant (*MATa*, *bet2-1* cells mate as efficiently as wild type in a quantitative mating test). In addition, the *dpr1(ram1)* mutant is not appreciably suppressed by the *BET2* gene when this is introduced on a high copy number vector. The same result was also

obtained in the reciprocal experiment. These findings imply that Bet2 may be a component of a novel modification pathway that recognizes carboxy-terminal consensus sequences that end in a CC motif rather than a CAAX box. As the inhibition of isoprenoid synthesis retards the growth of tumours in animal cells by preventing the attachment of Ras to membranes¹⁹, an understanding of the specificity of different protein prenyltransferases for their substrate may be useful in the therapeutic treatment of Ras-dependent tumours^{8,17,20,21}. □

Received 26 October 1990; accepted 22 March 1991.

- Barbacid, M. A. *Rev. Biochem.* **56**, 779–827 (1987).
- Segev, N., Mulholland, J. & Botstein, D. *Cell* **52**, 915–924 (1988).
- Bacon, R. A., Salminen, A., Ruohola, H., Novick, P. & Ferro-Novick, S. *J. Cell Biol.* **109**, 1015–1022 (1989).
- Baker, D., Wuestehube, L., Schekman, R., Botstein, D. & Segev, N. *Proc. natn. Acad. Sci. U.S.A.* **87**, 355–359 (1990).
- Salminen, A. & Novick, P. *Cell* **49**, 527–538 (1987).
- Bourne, H. *Cell* **53**, 669–671 (1988).
- Newman, A. & Ferro-Novick, S. *J. Cell Biol.* **105**, 1587–1594 (1987).
- Schafer, W. R. *et al. Science* **249**, 1133–1139 (1990).
- Powers, S. *et al. Cell* **47**, 413–422 (1986).
- Fujiyama, A., Matsumoto, K. & Tamanoi, F. *EMBO J.* **6**, 223–228 (1987).
- Peterson-Bjorn, S., Harrington, T. R. & Friesen, J. D. *Yeast* **6**, 345–352 (1990).
- Ruohola, H., Kabcenell, A. K. & Ferro-Novick, S. *J. Cell Biol.* **107**, 1456–1476 (1988).
- Groesch, M., Ruohola, H., Bacon, R., Rossi, G. & Ferro-Novick, S. *J. Cell Biol.* **111**, 45–53 (1990).
- Hancock, J. F., Magee, A. I., Childs, J. E. & Marshall, C. J. *Cell* **57**, 1167–1177 (1989).

- Molenaar, C. M. T., Prange, R. & Gallwitz, D. *EMBO J.* **7**, 971–976 (1988).
- Walworth, N. C., Goud, B., Kabcenell, A. K. & Novick, P. *EMBO J.* **8**, 1685–1693 (1989).
- Schafer, W. R. *et al. Science* **245**, 379–384 (1989).
- Goud, B., Salminen, A., Walworth, N. & Novick, P. *Cell* **53**, 753–768 (1988).
- Maltese, W. A., Defendi, R., Green, R. A., Sheridan, K. M., Donley, D. J. *clin. Invest.* **76**, 1748–1754 (1985).
- Goldstein, J. L. & Brown, M. S. *Nature* **343**, 425–430 (1990).
- Reiss, Y., Goldstein, J. L., Seabra, M. C., Casey, P. J. & Brown, M. S. *Cell* **62**, 81–88 (1990).
- Marshall, M. S., Gibbs, J. B., Scolnick, E. M. & Sigal, I. S. *Molec. cell. Biol.* **7**, 2309–2315 (1987).
- Carlson, M. & Botstein, D. *Cell* **28**, 145–154 (1982).
- Rose, M., Novick, P., Thomas, J., Botstein, D. & Fink, G. *Gene* **60**, 237–243 (1987).
- Goodman, L., Perou, C. M., Fujiyama, A. & Tamanoi, F. *Yeast* **4**, 271–281 (1988).

ACKNOWLEDGEMENTS. We thank S. Michaelis for informative discussions, P. Brenwald and S. Michaelis for a critical reading of the manuscript, J. Graf for technical assistance, N. Segev (anti-Ypt1), P. Novick (anti-Sec4) and J. Gibbs (anti-Ras) for antibodies, B. Schafer and J. Rine for strains. This work was supported by the National Cancer Institute and the Mathers Foundation (S.F.-N.).

Identification *in situ* and phylogeny of uncultured bacterial endosymbionts

Rudolf Amann, Nina Springer, Wolfgang Ludwig, Hans-Dieter Görtz* & Karl-Heinz Schleifer

Lehrstuhl für Mikrobiologie, Technische Universität München, Arcisstr. 21, 8000 München 2, Germany
* Zoologisches Institut der Universität, Schloßplatz 5, 4400 Münster, Germany

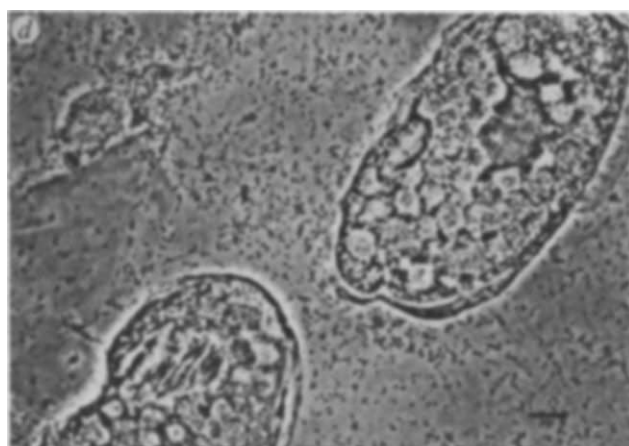
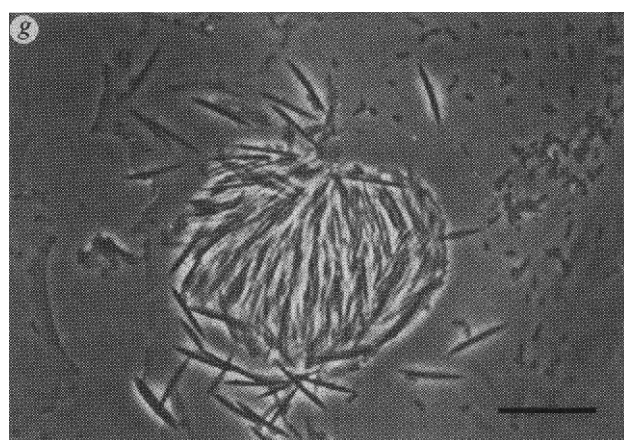
THE use of Koch's technique to isolate bacteria in pure cultures has enabled thousands of bacterial species to be characterized. But for the many microorganisms that have never been cultivated, DNA amplification *in vitro* using the polymerase chain reaction is now making their genes accessible^{1–3}. Here we use this technique to study bacteria of the genus *Holospora*, which live in ciliates⁴ and whose phylogenetic relationship has remained unknown because they are impossible to cultivate. Species of *Holospora* are highly infectious^{5–7} and live in the nuclei of their specific host cells: *H. elegans* and *H. undulata* infect micronuclei of *Paramecium caudatum*⁸, whereas *H. obtusa* infects the macronucleus in other strains of the same host species⁹; *Holospora* species have a common developmental cycle^{10–13}. We have amplified, cloned and sequenced gene fragments encoding ribosomal RNA of *H. obtusa*. The phylogenetic position of *H. obtusa* in the α group of *Proteobacteria* was determined by 16S rRNA sequence analysis. The sequences were then used to design species- as well as genus-specific rRNA hybridization probes, which enabled us to detect and differentiate individual cells of the endosymbionts *in situ*. The large amount of rRNA in the cells indicates a high physiological activity of the endosymbionts in the host nuclei.

Our approach to studying *H. obtusa* is summarized in the flow diagram of Fig. 1. It may be generally applicable for the study of ecosystems ill-defined by pure culture technique. Comparative rRNA sequence analysis is at present the best method for reconstructing phylogenetic relationships below the species level. We analysed the 16S rRNA of *H. obtusa* as detailed in the legend to Fig. 2. A phylogenetic affiliation was found between the newly obtained 16S rRNA sequence and the α subclass¹⁴

of the proteobacterial phylum¹⁵. Within this subclass no close relative of *H. obtusa* has so far been analysed at the rRNA level. Interestingly, this phylogenetic tree reveals a moderate relationship between *H. obtusa* and the intracellular parasites *Rickettsia*. Also the mitochondrial 16S rRNAs have a common phylogenetic root with members of the α subclass of *Proteobacteria*¹⁶.

To prove that the amplified and cloned sequences originated from the endonuclear symbionts and were not artefacts from contaminating bacteria that were used for feeding the ciliates, we performed *in situ* hybridizations with fluorescent oligonucleotide probes. This is the most direct way to link the sequences³ to a defined morphotype^{17–20}. On the basis of 16S rRNA analysis, the specific hybridization probe H16-23a (5'-TTCCACTTCTCTACCG-3') was designed to differentiate the newly obtained sequence from other bacterial 16S rRNA sequences. Fluorescently labelled, rRNA-targeted oligonucleotide probes enable individual bacterial cells to be detected^{18,19}. A specific probe labelled with tetramethylrhodamine was used along with a fluorescein-labelled probe complementary to all bacterial 16S rRNAs so far sequenced (5'-GCTGCCTCCCGTAGGAGT-3')²¹. This second probe served as a control for the presence and accessibility of bacterial 16S rRNA molecules within the host paramecia.

After hybridization, epifluorescence microscopy with a fluorescein-specific filter set revealed that the bacterial 16S rRNA signature sequence was present in the food vacuoles as well as in the macro- or micronuclei (Fig. 3). But the rhodamine-labelled specific probe H16-23a only hybridized to rod-shaped cells in the macro- or micronuclei of infected paramecia (Fig. 3). As many as several thousand *H. obtusa* cells were densely packed into the single macronucleus (up to 80 μ m in diameter), and several hundreds to thousands of *H. elegans* or *H. undulata* cells filled the micronuclei (one to three per cell with a diameter of 20 to 50 μ m). There were no suitable target sites within the *H. obtusa* 16S rRNA sequence for probes to distinguish the three closely related species from one another. We therefore sequenced a highly variable part of the 23S rDNA of *H. obtusa* and constructed a species-specific probe (Hobt56a 5'-AACACCCTACTTCTTTCG-3'). Applying this probe as described could differentiate between micro- and macronuclear endosymbionts in the host cells so that only *H. obtusa* was identified. This clearly demonstrated that we had amplified,



cloned and sequenced parts of the 16S and 23S rDNA molecules of *H. obtusa*.

Using rRNA-targeted oligonucleotide probes *in situ*, even more information of ecological importance can be retrieved. The amount of hybrid probe is directly correlated to the cellular rRNA content, which itself is a measure of physiological activity¹⁸. By this criterion most of the endosymbionts are highly active in their habitat, because hybridization of the bacterial probe is as strong (or stronger) with endonuclear *Holospira* cells as it is with surrounding *Enterobacter aerogenes* which are

added as feed from a logarithmic culture.

We have described an approach that allows a definitive correlation to be made between an unknown sequence obtained from a complex environment, and cells living in that environment. The techniques should help microbial taxonomists and ecologists learn more about organisms that have so far proved impossible to culture. This should be particularly useful, as it is thought that only a small part of the microbial diversity has so far been discovered and studied^{1,2,22}. □

Received 4 February; accepted 18 March 1991.

1. Giovannoni, S. J., Britschgi, T. B., Moyer, C. L. & Field, K. G. *Nature* **345**, 60–63 (1990).
2. Ward, D., Weller, R. & Bateson, M. M. *Nature* **345**, 63–65 (1990).
3. Saiki, R. K. *et al. Science* **239**, 487–491 (1988).
4. Preer, J. R. Jr & Preer, L. B. *Int. J. Syst. Bact.* **32**, 140–141 (1984).
5. Hafkine, M. W. A. *Rev. Inst. Pasteur* **4**, 148–162 (1890).
6. Gromov, B. V. & Ossipov, D. V. *Int. J. Syst. Bact.* **31**, 348–352 (1981).
7. Preer, J. R. Jr & Preer, L. B. In *Bergey's Manual of Systematic Bacteriology* (eds Sneath, P. H. A., Mair, N. S., Sharpe, M. E. & Holt, J. G.) 795–813 (Williams & Wilkins, Baltimore, 1984).
8. Görtz, H. D. & Dieckmann, J. *Protistologica* **16**, 591–603 (1980).
9. Ossipov, D. V., Skoblo, I. I. & Rautian, M. S. *Acta protozool.* **14**, 263–280 (1975).
10. Görtz, H. D. *Int. Rev. Cytol.* **102**, 169–213 (1986).
11. Görtz, H. D., Ahlers, H. & Robenek, H. *J. gen. Microbiol.* **135**, 3079–3085 (1989).
12. Görtz, H. D., Lellig, S., Miosga, O. & Wiemann, M. *J. Bact.* **172**, 5664–5669 (1990).
13. Fujishima, M., Nagahara, K. & Kojima, Y. *Zool. Sci.* **7**, 849–860 (1990).
14. Woese, C. R. *Microbiol. Rev.* **51**, 221–271 (1987).
15. Stackebrandt, E., Murray, R. G. E. & Trüper, H. G. *Int. J. Syst. Bact.* **38**, 321–325 (1988).

16. Yang, D., Oyaizu, Y., Oyaizu, H., Olsen, G. J. & Woese, C. R. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4443–4447 (1985).
17. Edman, J. C. *et al. Nature* **334**, 519–522 (1988).
18. Giovannoni, S. J., DeLong, E. F., Olsen, G. J. & Pace, N. R. *J. Bact.* **170**, 720–726 (1988).
19. DeLong, E. F., Wickham, G. S. & Pace, N. R. *Science* **243**, 1360–1363 (1989).
20. Amann, R. I., Krumholz, L. & Stahl, D. A. *J. Bact.* **172**, 762–770 (1990).
21. Amann, R. I. *et al. Appl. environ. Microbiol.* **56**, 1919–1925 (1990).
22. Torsvik, V., Goksoyr, J. & Daee, F. L. *Appl. environ. Microbiol.* **56**, 782–787.
23. Hori, H. & Osawa, S. *Proc. natn. Acad. Sci. U.S.A.* **76**, 381–385 (1979).
24. Saitou, N. & Nei, M. *Molec. Biol. Evol.* **4**, 406–425 (1987).
25. Neefs, J. M., Van de Peer, Y., Hendriks, L. & De Wachter, R. *Nucleic Acids Res.* **18**, 2237–2318 (1990).
26. Dryden, S. C. & Kaplan, S. *Nucleic Acids Res.* **18**, 7267–7277 (1990).
27. Schmidt, H. J., Freiburg, M. & Görtz, H. D. *Microbios.* **49**, 189–197 (1987).
28. Chen, E. Y. & Seeburg, P. H. *DNA* **4**, 165–170 (1985).
29. Woese, C. R., Kandler, O. & Wheelis, H. L. *Proc. natn. Acad. Sci. U.S.A.* **87**, 4576–4579 (1990).

ACKNOWLEDGEMENTS. This work was supported by the Deutsche Forschungsgemeinschaft.

Key residues involved in calcium-binding motifs in EGF-like domains

P. A. Handford*, M. Mayhew*, M. Baron†‡, P. R. Winship*§, I. D. Campbell†‡ & G. G. Brownlee*‡

* Sir William Dunn School of Pathology, † Department of Biochemistry and ‡ Oxford Centre for Molecular Sciences, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

MANY extracellular proteins with diverse functions contain domains similar to epidermal growth factor (EGF), a number of which have a consensus Asp/Asn, Asp/Asn, Asp*/Asn*, Tyr/Phe (where the asterisk denotes a β -hydroxylated residue)¹. These include the coagulation factors IX and X, proteins with two EGF-like domains, the first of which contains the consensus residues². The first EGF-like domain of human factor IX contains a calcium-binding site, which is believed to be responsible for one of the high-affinity sites detected in this protein³. Similar results have been obtained for bovine factor X⁴. We have now used protein engineering and ¹H-NMR techniques to investigate the importance of individual consensus residues for ligand binding. Measurement of a calcium-dependent Tyr 69 shift³ in the isolated first EGF-like domain from human factor IX demonstrates that Asp 47, Asp 49, and Asp 64 are directly involved in this binding. Gln 50, whose importance has previously been overlooked, is also involved in this binding. Two mutations⁵ in this domain, Asp 47→Glu, and Asp 64→Asn, present in patients with haemophilia B, reduce calcium binding to the domain >4-fold and >1,000-fold, respectively. Furthermore, the defective calcium binding of Asn 64 can be partially rescued by the compensatory mutation Gln 50→Glu. This latter mutation, when introduced singly more than doubles the affinity of the domain for calcium. This study thus defines residues involved in a new type of calcium-binding site and provides strong circumstantial evidence for calcium-binding motifs in many

extracellular proteins, including the developmentally important proteins of *Drosophila*, notch, delta and crumbs^{1,6–8}.

The sequence of the first EGF-like domain from human factor IX is shown in Fig. 1a. A structural model of this domain, based on the ¹H-NMR solution structure of human EGF⁹ (Fig. 1b), shows the predicted arrangement and the proximity of the consensus residues Asp 47, Asp 49, Asp 64 and Tyr 69 to one another. To test the importance of each carboxylate for calcium binding, mutations were introduced into the domain (see Table 1). These include two mutations identified in haemophilia B patients (referred to as Oxford d1 and d3)⁵ which have the point mutations Asp 64→Asn and Asp 47→Glu respectively, and two mutations previously studied in genetically engineered factor IX proteins¹, Asp 49→Glu and Asp 64→Lys, which have not been detected in patients. Mutant domains (residues 46–84 of human factor IX) were expressed using a yeast secretion system³ (for details of cloning procedures see legend to Table 1), refolded and purified by HPLC. Each mutant domain was analysed by ¹H-NMR and its spectrum compared with the wild-type domain to determine whether the introduced mutation had disrupted structure. Figure 2 shows the one-dimensional NMR spectrum at pH 7.4 of the aromatic region of four of the mutant domains compared with the wild-type domain. All show striking similarity to the wild-type profile, indicating that the introduced mutations have not disrupted the overall domain structure—a prerequisite for these calcium binding studies. All other mutant domains tested showed similar profiles. Calcium (as CaCl₂) was then titrated into each protein sample in the absence of other ions and the movement of the calcium-dependent Tyr 69 resonance (arrowed in Fig. 2) recorded. A subsequent set of calcium titrations was performed at constant ionic strength (*I* = 0.15, equivalent to physiological ionic strength) to exclude this as a variable affecting calcium binding. The change in chemical shift of the Tyr 69 resonance was plotted against free calcium concentration to obtain a dissociation constant (Fig. 3a). Dissociation constant (*K_d*) values are summarized in Table 1.

In mutants Asp 47→Glu, Asp 49→Glu and Asp-64→Glu, or Lys, or Asn, there is a large reduction in the affinity (increase in *K_d*) of the domain for calcium, at both variable and constant ionic strength. Asp 49 and Asp 64 seem to be more important ligands than Asp 47 as in the examples where conservative

§ Present address: Department of Medicine and Pharmacology, University of Sheffield, Royal Hallamshire Hospital, Sheffield, UK.

mutations (Asp → Glu) have been introduced at each of the three carboxylate residues, the affinity of these mutants for calcium is most severely affected (see Table 1). In addition, there are no marked pH effects on the K_d for these mutants over the range measured (pH 6.5–8.5, Fig. 3b). This argues against the reduced affinity being caused by an indirect effect of the mutation on the pH optimum for calcium binding. Two other mutations introduced at Asp 64 (Asp → Asn, Asp → Lys) seem to have an even greater inhibitory effect on calcium binding. No binding was detected in the case of the Asp 64 → Lys mutation and only a slight movement of the Tyr 69 resonance was detected at 250 mM Ca^{2+} for the Asp 64 → Asn mutation. In summary, these data establish the critical importance of the carboxylate residues Asp 47, 49 and 64 for calcium binding.

The virtual absence of calcium binding to the Asp 64 → Asn mutant domain is of interest as many examples of EGF-like domains are known with an Asn residue in the equivalent position to Asp 64 of factor IX¹. Closer examination of these EGF-like domain sequences, however, reveals that, in general, Asp at position 64 is associated with either Gln (type I consensus, see Fig. 1a) or Glu (type III consensus) at position 50, whereas Asn is associated with Glu rather than Gln (type II consensus). This suggested that the residue at position 50 in factor IX may be important in calcium binding. Therefore we constructed a double mutant Asp 64 → Asn, Gln 50 → Glu, as well as the single mutant Gln 50 → Glu, to test if we could rescue calcium binding which is absent in the mutant Asp 64 → Asn. These represent the type II and type III combinations of consensus residues (see Fig. 1a) within a factor IX EGF-domain background. In each we could demonstrate improved calcium binding at constant ionic strength (Asp 64 → Asn, Gln 50 → Glu, $K_d \sim 10$ mM; Gln 50 → Glu, $K_d = 0.5$ mM; Table 1 and Fig. 3a). These mutants, together with the wild-type domain, thus indicate that all three variations of the consensus sequence (type I, II, III) in a factor IX background support calcium binding. Recent two-dimensional ¹H-NMR structural analysis of the first EGF-like domain from factor IX has confirmed that Gln 50 is close in space to Tyr 69 and thus in the vicinity of the calcium-binding region¹⁰. The rescue of calcium binding to the Asp 64 → Asn mutant domain by the introduction of a second mutation Gln 50 → Glu was pH-independent (over the range measured, pH 6.5–8.5; Fig. 3b). This provides strong evidence for the

TABLE 1 Values of K_d for Ca^{2+} binding to mutant domains

Mutation in FIX EGF domain	K_d at pH 7.4 (mM)		Specific activity* Intact factor IX (%)
	$I = \text{variable}^\dagger$	$I = 0.15$	
Wild type	0.25	1.8	100
Asp 47 → Glu (Oxford d3)	2.5	8.9	1 ⁵
Asp 49 → Glu	4.0	67	8 ¹
Asp 64 → Glu	4.0	105	—
Asp 64 → Lys	Undetectable	—	2 ¹
Asp 64 → Asn (Oxford d1)	>250	—	3 ⁵
Asp 64 → Asn, Gln 50 → Glu	1.5	10.2	—
Gln 50 → Glu	<0.1 $\ddagger$	0.5	—

Mutations were introduced into the factor IX EGF-like domain coding sequence by standard protein engineering techniques¹⁶ or by the polymerase chain reaction procedure¹⁷. In the latter case oligonucleotides were used to amplify a region of genomic DNA corresponding to nucleotides 10,391–10,504 of the factor IX sequence¹⁸. Amplifications were performed on a Cetus DNA thermal cycler using an initial denaturation step of 95 °C for 7 min, followed by 30 cycles of 91 °C for 1 min, 55 °C for 1 min, 70 °C for 3 min. Buffer and enzyme additions were as described previously¹⁷. Amplified DNA was directly sequenced before cloning into the yeast expression vector¹⁹. Details of the yeast secretion method used to express the domains have been reported elsewhere³. Mutant domains (0.5–2 mg) were refolded before analysis²⁰. N-terminal sequence analysis of the domains by Edman degradation confirmed that all contained the correct mutations. The domains produced by the yeast biosynthetic route did not contain the post-translational modifications present in human factor IX, specifically a β -hydroxyaspartic acid at position 64²¹, and an *O*-linked carbohydrate modification on Ser 53²²; however, neither of these modifications is necessary for calcium binding to the wild-type domain³. —, Not done.

* Specific activity is defined as clotting activity/antigen where factor IX antigen and clotting activities are expressed as a percentage of that antigen or clotting activity found in normal human plasma.

[†] The K_d values obtained when $I = \text{variable}$ (in the absence of NaCl) should be regarded as apparent K_d values.

[‡] 0.1 mM represents the lower limit of our experimental accuracy for the quantitative determination of K_d .

participation of Glu 50 in direct ligand binding in this domain. In the case of the single mutant Gln 50 → Glu domain we cannot, however, rule out the possibility that the introduction of an additional ionizable group has indirectly increased affinity by

a		47		49 50		64	
		D	G	D	Q	D	
I	HFIX (47–84)	D	G	D	Q	D	DINSYECWCPFGFEG KNCLE
	HFX (46–84)	D	G	D	Q	D	GLGEYTCCTLEGFEG KNCLEF
II	NOTCH (449–487)	D	I	D	E	N	TPGSYRCNCSQGFPG PRCET
	CRUMBS (648–688)	D	V	D	E	N	ERGSYKCYCTPGFTG VHCDS
	DELTA (529–566)	D	I	D	E	N	RVNSFECVCANGFRG KQCDE
III	NOTCH (526–563)	D	I	D	E	D	KINGFKCSALGFTG ARCQI
	CRUMBS (388–426)	D	T	D	E	D	RINGFSCDCSGTGYTGAFCQT

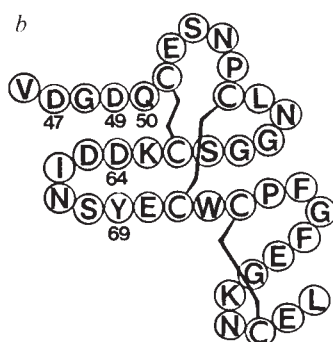


FIG. 1 a, Sequence comparison of some EGF-like domains with the consensus Asp/Asn, Asp/Asn, Asp*/Asn*, Tyr/Phe. For a more detailed list see ref. 1. Residues Asp 47, Asp 49, Gln 50 and Asp 64 (numbering as in factor IX), which are implicated in calcium binding, are boxed. Three variations of the consensus are shown. Type I: Asp, Asp, Gln, Asp (for example human factor IX, human factor X); type II: Asp, Asp, Glu, Asn (for example notch 449–487, crumbs 648–688, delta 529–566); type III: Asp, Asp, Glu, Asp (for example notch 526–563, crumbs 388–426). Sequences for notch, delta, and crumbs were obtained from published data^{6–8}. b, The first EGF-like domain from human factor IX (residues 46–84) modelled on the solution structure of human EGF⁹. Residues referred to in the text are numbered. Single-letter amino-acid code is used.

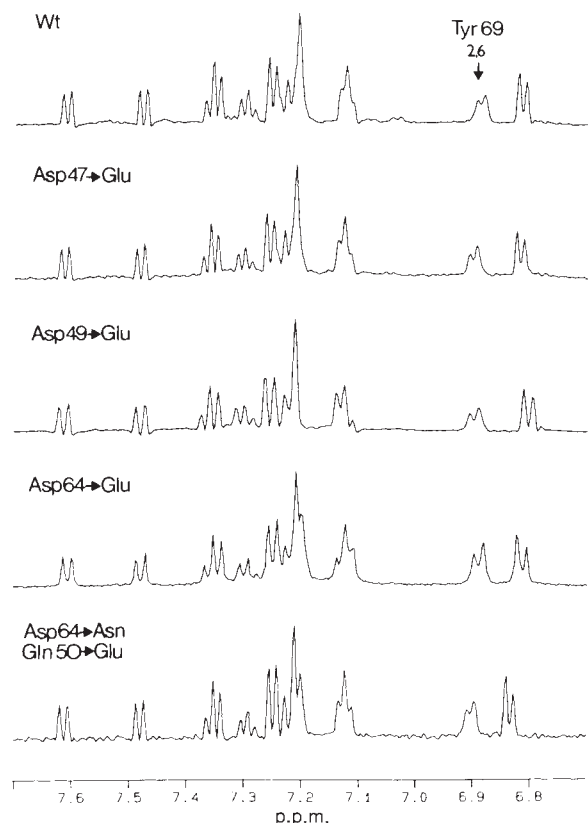


FIG. 2 A comparison of one-dimensional ^1H -NMR spectra of wild-type (Wt) and mutant factor IX EGF-domains at pH 7.4, 303 K, in D_2O , in the absence of Ca^{2+} and other ions showing the aromatic region of the spectrum. Spectra were collected on a Bruker AM500 MHz spectrometer. Assignment of resonances in this region have been described previously³. The Tyr 69, 2,6 proton resonance used to measure calcium binding to the domain is indicated with an arrow.

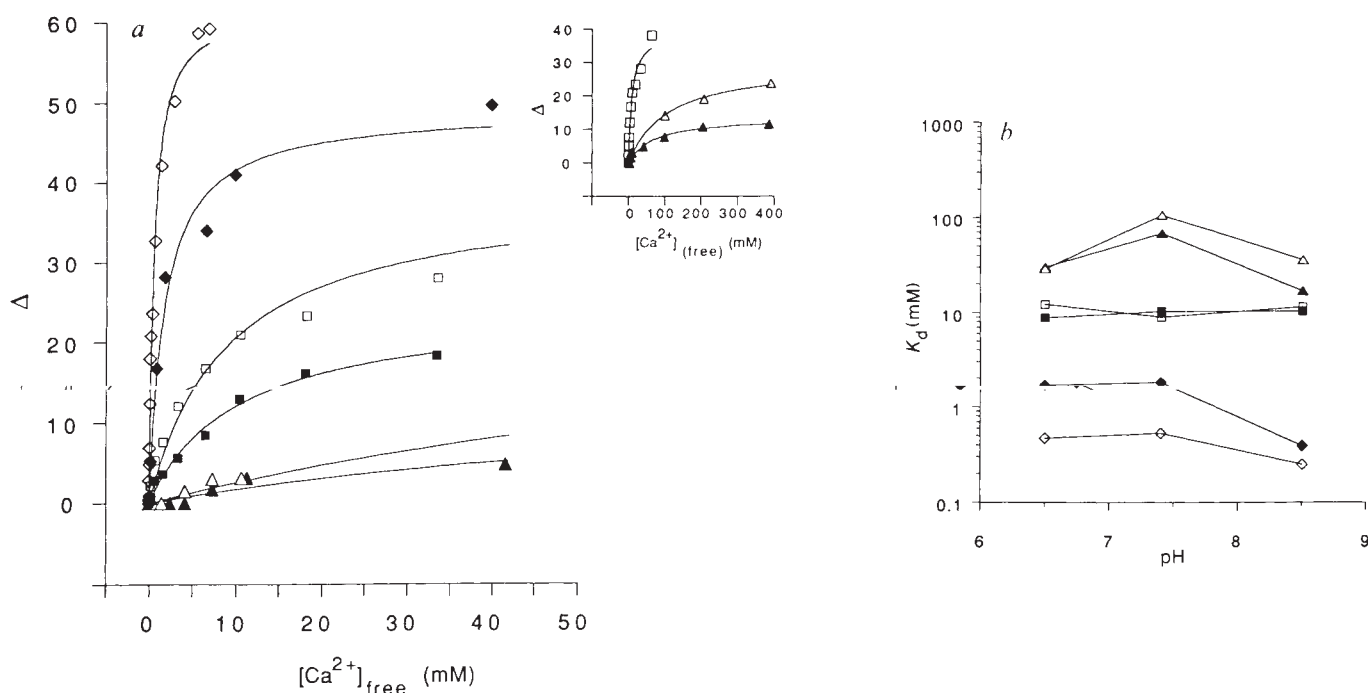


FIG. 3 *a*, The change in chemical shift (Hz) of the Tyr 69, 2,6 proton resonance (Δ) at pH 7.4, 303 K and constant ionic strength ($I=0.15$) plotted against free calcium. Gln 50 $\rightarrow$ Glu ($\diamond$), wild type ($\blacklozenge$), Asp 47 $\rightarrow$ Glu ($\square$), Asp 64 $\rightarrow$ Asn, Gln 50 $\rightarrow$ Glu ($\blacksquare$), Asp 49 $\rightarrow$ Glu ($\blacktriangle$), Asp 64 $\rightarrow$ Glu ($\triangle$). Protein samples were dissolved in D_2O and the pH adjusted to 7.4. For calcium titrations carried out at constant ionic strength, NaCl was added to the protein sample to give a final concentration of 0.15 M ($I=0.15$), which approximates to physiological ionic strength. Stock CaCl_2 (at pH 7.4) was adjusted with NaCl to give a total ionic strength of 0.15 using the formula $I=\frac{1}{2}\sum cz^2$, before addition to the sample. Curves were fitted according to the equation $\Delta=\Delta_0[\text{Ca}_{\text{free}}]/(K_d+[\text{Ca}_{\text{free}}])$ by standard linear regression analysis. Free calcium

was calculated from the equation $[\text{Ca}_{\text{free}}]=[\text{Ca}_{\text{total}}]-\Delta/\Delta_0[\text{protein}]$ (assuming one binding site), where Δ/Δ_0 is the fractional change in the chemical shift of the Tyr 69 resonance, in order to correct for the relatively high amounts of protein used in the experiment. For mutants Asp 64 $\rightarrow$ Glu, Asp 49 $\rightarrow$ Glu, Asp 47 $\rightarrow$ Glu, data points obtained at ionic strength >0.15 were included for the calculation of K_d values. The inset graph shows the complete calcium titration data for these mutants. *b*, The pH dependence of calcium binding to factor IX EGF-like domains. Calcium titrations were carried out at constant ionic strength ($I=0.15$) at pH 6.5, 7.4, 8.5, as detailed above; symbols as for Fig. 3*a*.

altering the pH optimum for calcium binding, as the wild type binds calcium with a similar affinity to Gln 50 → Glu at pH 8.5 (Fig. 3b). Despite this, our binding data, together with the observed sequence conservation discussed above, clearly involve residue 50, either directly or indirectly, with calcium binding.

Our data suggest that the reduced clotting activities in mutant factor IX samples, and thus the phenotypic effect of mutations in the haemophilia B patients Oxford 31 and 33, are due to reduced calcium binding to the isolated domain (Table 1). It is unlikely that the reduction in clotting activity is due to gross perturbation of the EGF domain as NMR analysis of the isolated domains showed them to be virtually identical to the wild type in the absence of calcium (Fig. 2).

The two reported high-affinity calcium-binding sites in Gla-domainless factor IX, measured in the presence of 0.1 M NaCl, have dissociation constants of $\sim 50 \mu\text{M}^{11}$. We have previously argued that the K_d observed for the isolated domain in NMR experiments corresponds to one of the calcium-binding sites in Gla-domainless factor IX, although the binding to the isolated domain is weaker³. Similar results have been obtained for the isolated bovine factor X domain⁴. Additional contacts outside the first EGF-like domain presumably increase the affinity of the site.

The precise role of calcium in the EGF-like domain is unclear. Calcium bound to the domain may stabilize a conformation required for protein-protein interactions, a common feature of proteins containing these domains^{1,12-15}. More speculatively, if two proteins with these domains were required to interact, then calcium ions might bridge two EGF-like domains, with specificity being controlled by additional contacts outside this region. It has recently been demonstrated that notch and delta, two proteins involved in the neurogenic developmental pathway of *Drosophila*, directly interact in a calcium-dependent manner¹². This is of interest as notch has 36 EGF-like domains and delta has 9, of which 6 and 1, respectively, contain calcium-binding sequences of the type studied here^{6,7}. Although direct interaction through these domains has not been demonstrated, the fact that the interaction is calcium-dependent is highly suggestive. Furthermore, the *Drosophila* crumbs protein has 5 out of 30 EGF-like domains with calcium-binding motifs demonstrated in this study⁸. It is possible that calcium-dependent interactions form an integral part in the role of this protein in epithelial organization. □

Received 18 March; accepted 2 April 1991.

- Rees, D. J. G. *et al.* *EMBO J.* **7**, 2053-2061 (1988).
- Furie, B. & Furie, B. C. *Cell* **53**, 505-518 (1988).
- Handford, P. A. *et al.* *EMBO J.* **9**, 475-480 (1990).
- Persson, E. *et al.* *J. Biol. Chem.* **264**, 16897-16904 (1989).
- Winship, P. & Dragon, A. C. *Br. J. Haematol.* **77**, 102-109 (1991).
- Wharton, K. A., Johansen, K. M., Xu, T. & Artavanis-Tsakonas, S. *Cell* **43**, 567-581 (1985).
- Vassin, H., Bremer, K. A., Knust, E. & Campos-Ortega, J. *EMBO J.* **6**, 3431-3440 (1987).
- Tepass, U., Theres, C. & Knust, E. *Cell* **61**, 787-799 (1990).
- Cooke, R. M. *et al.* *Nature* **327**, 339-341 (1987).
- Baron, M. thesis, Univ. Oxford (1990).
- Morita, T. & Kiesel, W. *Biochem. biophys. Res. Commun.* **130**, 841-847 (1985).
- Fehon, R. G. *et al.* *Cell* **61**, 523-534 (1990).
- Öhlin, A. *et al.* *J. Biol. Chem.* **263**, 19240-19248 (1988).
- Kurosawa, S., Stearns, D. J., Jackson, K. W. & Esmon, C. T. *J. Biol. Chem.* **263**, 5993-5996 (1988).
- Villiers, C. L., Arlaud, G. J. & Colomb, M. G. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4477-4481 (1985).
- Kunkel, T. *Proc. natn. Acad. Sci. U.S.A.* **82**, 488-492 (1985).
- Saiki, R. K. *et al.* *Science* **239**, 487-491 (1988).
- Yoshitake, S., Schach, B. G., Foster, D. C., Davie, E. W. & Kurachi, K. *Biochemistry* **24**, 3736-3750 (1985).
- Winship, P. R. *Nucleic Acids Res.* **17**, 1266 (1989).
- Jaenicke, R. & Rudolph, R. in *Protein Structure: A Practical Approach* (ed. Creighton, T. E.) 208-209 (IRL, Oxford, 1989).
- Fernlund, P. & Stenflo, J. *J. Biol. Chem.* **258**, 12509-12512 (1983).
- Nishimura, H. *et al.* *Thromb. Haem.* **62**, 17 (1989).

ACKNOWLEDGEMENTS. We thank A. Willis and D. Lamont for carrying out N-terminal sequence and amino-acid analyses. We thank R. Davies for many helpful discussions. P.H. is supported by the MRC (G.G.B.). P.R.W. is supported by the Royal Society Alan Johnston Lawrence and Moseley research fellowship. This is a contribution from the Oxford Centre for Molecular Sciences, which is supported by the SERC and MRC.

nature is available in microform



University Microfilms

International reproduces this publication in microform: microfiche and 16mm or 35mm film. For information about this publication or any of the more than 13,000 titles we offer, complete and mail the coupon to: University Microfilms International, 300 N. Zeeb Road, Ann Arbor, MI 48106. Call us toll-free for an immediate response: 800-521-3044. Or call collect in Michigan, Alaska and Hawaii: 313-761-4700.

☐ Please send information about these titles:

Name _____

Company/Institution _____

Address _____

City _____

State _____ Zip _____

Phone (____) _____

University
Microfilms
International

Controlling laboratory vibrations

Patrick Wong

Tabletop vibrations, when not controlled, can adversely affect sensitive instrumentation. The sources of these laboratory vibrations and various vibration control methods are discussed.

CRITICAL biomedical experiments can be seriously affected by vibrations. When tabletop vibrations are not controlled, sensitive instrumentation is affected and research results are often seriously degraded. And, in applications such as electrophysiology, microinjection and cell manipulation, tabletop vibrations can cause complete failure of the experiment. For example, in precision work, such as the study of cell signalling in living brain slices conducted by Stephen Smith at Stanford University, even micron-scale relative motion between tabletop components can mean experimental failure. These minute vibrations can shake electrodes out of cells or they can cause mechanical tip displacement, which may rupture cells and make it necessary to repeat the experiment. As a result, it has become important to design worktables for the biomedical laboratory that are capable of providing a level of vibration control that allows ultraprecision.

There are five primary sources of disturbances that affect mechanical alignment in precision worktables. These include: ground or floor vibrational inputs; airborne vibrations (acoustic); vibrations generated by equipment or apparatus within the system; load changes of quasi-static forces that act on the system; and thermal changes such as heat source or ambient temperature changes. Although no vibration control system can completely eliminate every source of instability, one fact is clear: the better the vibration control, the better the equipment performs.

What are the alternatives?

The key to minimizing the effect of vibrations on sensitive equipment is to create a worktable situation where all parts of the system are moving together, rather than shifting around relative to each other. To accomplish this stability, the mounting surface of the worktable must have a high degree of rigidity and it must be isolated from environmental vibrations. Although a variety of crude vibration isolation methods (that is, the use of tennis balls positioned under cement blocks) have been tried in many laboratories, three main vibration control technologies have become dominant: (1) granite blocks, (2) aluminium honeycomb core tables with broadband, visco-elastic dampers, and (3) steel honeycomb cores with tuned-spring mass hydraulic dampers.

As critical experiments such as video microscopy, electrophysiology and ultra-microscopy require interferometric accuracy

from all instruments used on the tabletop, vibration control worktables must be designed to meet a demanding set of performance requirements. Granite slabs, the conventional solution for isolating vibrations, have been tried in some laboratories. However, they are bulky and have many drawbacks in crowded biomedical laboratories. For example, they can weigh a ton or more, making structural support and size major obstacles. They are also limited in their ability to provide the extremely high level of vibration control needed to perform delicate experiments, such as the study of living brain slices mentioned earlier, as they are easily excited by both acoustic and mechanically transmitted vibrations.

While it may seem difficult to induce vibrations in a block weighing thousands of pounds, granite cannot match the dynamic rigidity (the ability to damp, or resist the deformation that results from internal and external vibrational inputs) of a far lighter damped steel-honeycomb construction. Mass that does not contribute to stiffness only decreases the resonant frequencies of a structure, thus increasing displacements. Granite blocks subjected to vibrations have pronounced resonance peaks. Also, as the internal damping of granite is low, the material continues to vibrate, or 'ring', long after the stimulus ceases. These micron-scale motions reduce the accuracy, precision and repeatability of critical experiments.

The use of lightweight honeycomb structures for worktables offers a greater level of vibration control as well as a much higher level of flexibility for the biomedical laboratory where space and transportability are important considerations. The core structure of a honeycomb table provides a high strength-to-weight ratio and a stiffer, more stable platform. The low weight of honeycomb structures keeps the resonant frequency high — minimizing large-amplitude, low-frequency displacements. Additionally, internal damping dissipates the vibrational energy of the tabletop by converting small amplitude mechanical vibrations to heat. This lowers resonant motion peaks across the entire vibration frequency spectrum. Because every table has dominant bending and torsional modes (each of which has a characteristic frequency and table deformation shape), optimal damping of these resonant modes vastly improves the stability of the table. Two primary technologies are used for damping: broadband, visco-elastic dampers and tuned spring mass hydraulic dampers.

Tuning out resonances

The broadband, visco-elastic dampers indiscriminately absorb a moderate amount of energy over a wide range of frequencies. But, as they are not tuned to damp dominant table resonances, they are much less effective than tuned dampers. Additionally, the damping characteristic of the visco-elastic material changes over time, making future vibration control performance unpredictable.

The use of multiple tuned dampers, each individually tuned to minimize table motion at specific resonance modes, is a much more effective solution (see Fig. 1). Tuned dampers concentrate damping where it is needed most, at the frequencies of the dominant resonance modes. Because broadband dampers are designed to provide moderate damping over a wide range of frequencies, they are not as effective at damping the dominant modes of vibration.

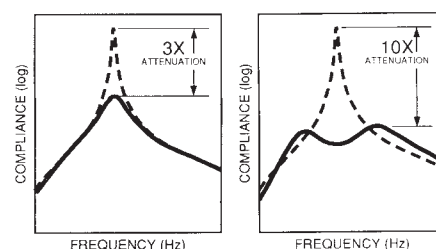


FIG. 1 Tuned dampers (left) concentrate damping at the frequencies of dominant resonance modes. As broadband dampers (right) are designed to provide moderate damping over a wide range of frequencies, they are not as effective at damping the dominant modes of table vibration.

Although honeycomb cores may be constructed from either steel or aluminium, steel typically provides greater vibration control. The comparison is best illustrated by looking at compliance curves (Fig. 2). Basically, compliance curves show the displacement amplitude of a point on a body per unit of impulse force applied. The greater the compliance, the more easily the structure moves as a result of applied force. The compliance curve for the steel tabletop in figure 2 demonstrates that the table bending with the same force is about $1.8 \times 10^{-5} \text{ mm N}^{-1}$, or roughly a factor of 11 more rigid than the standard aluminium honeycomb tabletop of the same size.

Isolating floor vibrations

Most biomedical laboratories are plagued with floorborne vibrations resulting from, for example, elevators, compressors, air con-

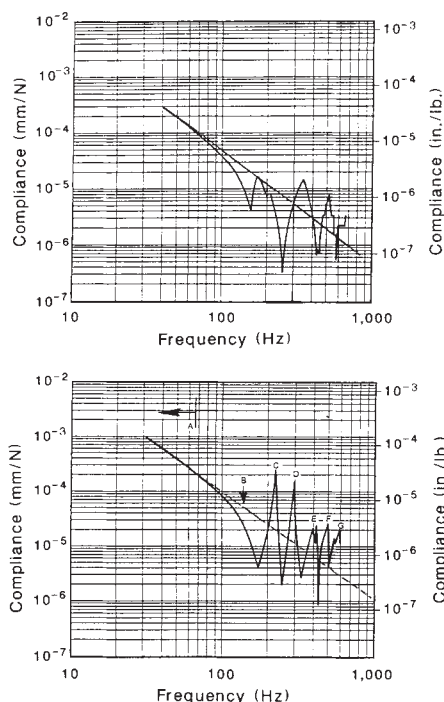


FIG. 2 The compliance curve for the steel tabletop (top) shows that the table bending with the same force is a factor of 11 more rigid than the standard aluminium tabletop (bottom) of the same size.

ditioners, and transformers, causing the table structure to resonate. As a result, the tabletop must be isolated from these 'resonance' vibrations as well as other tabletop vibrations. Of the main methods of isolating floorborne vibrations, pneumatic isolators offer the greatest level of vibration control. This is because they combine the fast 'roll-off' of the simple harmonic oscillator at vibration frequencies above isolator resonance and the low amplification of the damped harmonic oscillator near resonance.

In a pneumatic isolation system, the isolated mass is supported by a piston that rests on a flexible rolling diaphragm. Under the diaphragm are two pressurized air chambers connected by a small orifice. Air moves from the upper to the lower chamber through the orifice, dissipating energy and reducing amplification at the isolator's natural frequency.

Vibration control systems that are capable of interferometric accuracy are not only an important consideration in the biomedical laboratory, they are essential. When conducting experiments, that require ultraprecision, the floor, air and other room vibrations found in a typical laboratory can cause research delays or even experimental failure. As a result, the lightweight steel-honeycomb core worktable with pneumatic isolators has emerged as a useful vibration control device for the biomedical laboratory. □

Patrick Wong is at Newport Bio-Instruments, 18235 Mt Baldy Circle, P.O. Box 8020, Fountain Valley, California 92728-8020, USA. For more information, fill in reader service number 100.

Made to measure

A method for the dynamic *in situ* measurement of cell growth and viability, a portable photosynthesis analysis system, and software for collecting and analysing behavioural data — new aids for quantitative analysis.

ADAM Hilger, the publishing arm of the Institute of Physics has launched two new books in the series on Measurement Science and Technology (*Reader Service No. 101*). The first of the two books, entitled *SQUIDS, the Josephson Effects and Superconducting Electronics* by J.C. Gallop, outlines the development of superconducting electronics, and describes the fundamental principles, superconducting devices and their applications. In addition, practical tips and hints are provided for researchers using SQUIDS in their studies. The book costs £45 (UK). The second book is entitled *Uncertainty, Calibration and Probability: The Statistics of Scientific and Industrial Management* by C.F. Dietrich. Given that all measurements are subject to error, no quantity can be known exactly and, hence, any measurement has a probability of lying within a certain range, the IOP says this £90 (UK) book gives a comprehensive treatment of the statistics and methods of estimating these calibration uncertainties.

The CellStat system from ONCOtherapeutics provides a method of continuously monitoring cell proliferation and viability in culture, without disrupting important homotypic and heterotypic cell-cell relationships (*Reader Service No. 102*). CellStat technology uses a biosensor lid that interfaces with a standard 24-well tissue culture plate to measure minute electrochemical changes in cultured cells. The conductivity of the media, which is affected by cell density and viability, is monitored and the signals integrated by computer. The company says the CellStat system has been used to monitor the growth of normal and neoplastic cell types, including a variety of human colon cancer cells, and to assess the cytostatic and cytotoxic effects of the chemotherapeutic agents 5-fluorouracil and methotrexate. Cell death due to cytotoxic agents was dynamically and continuously monitored and the results depicted graphically, yielding specific dose-response curves for each cell type. The basic unit costs \$37,500 (US) and includes signal processing PC, modem, logic system, measurement system, enhanced graphics display unit, data acquisition, data analysis and data display software. The BioProbe lids cost \$50 (US) each.

A fully portable, battery-operated system for the measurement of photosynthesis and transpiration has been developed by the Analytical Development Company (*Reader Service No. 103*). The LCA-2 System, which consists of an infrared analyser, air-supply

unit, data logger and leaf chamber, provides information on photosynthetic rate, stomatal conductance, transpiration rate, humidity and CO₂ concentration. The leaf chamber incorporates sensors to measure humidity, temperature and photosynthetic active radiation. Four standard leaf-chamber designs are available, each manufactured for different plant species.

Research aids

Noldus Information Technology has developed Version 2.0 of the Observer software package, which turns an IBM PC or laptop computer into a powerful tool for recording the frequency and duration of behavioural events (*Reader Service No. 104*). It can also program various handheld computers for use as event recorders in the field.



Observational research software.

When using Observer, the keys of the computer keyboard are redefined to represent events (for example, w = walking, s = sitting). As many as 92 keys can be designated as events, says Noldus. Events can have one or two 'modifiers' to indicate the scope of an event, which can be useful when studying social interactions. The program also allows grouping of events in classes and distinction between mutually exclusive versus non-exclusive, and duration versus frequency events. In addition, up to 40 independent variables (for example, experimental conditions, environmental variables) can be specified. The Observer can create output files in a format that is suitable for importation into spreadsheet or statistics programs. The software is supplied either as a Base Package for IBM-compatible PCs, or as various Support Packages for non-IBM-compatible computers.

The four-channel, small animal exercise treadmill from Columbus Instruments allows for the simultaneous measurement of oxygen consumption of four mice or small rats under variable exercise conditions

ADVERTISEMENTS

Teflon^{*} Coated and Bare Wire from

MEDWIRE

Medwire provides the highest quality platinum, platinum alloy, silver and stainless steel wire in both single and multistrand configurations.

For prompt delivery or additional information, please contact:

MEDWIRE, an affiliate of Sigmund Cohn Corp.
121 So. Columbus Ave. Mount Vernon, NY 10553
Tel: (914) 664-5300 Fax: (914) 664-5377

*Reg. TM of E. I. DuPont & Co



Reader Service No.25

ACTIVITY MONITOR

Video tracking system, for water-maze, open field, radial maze etc
In the U.S. 1-800-225-9261

HVS IMAGE

Ormond Crescent

Hampton TW12 2TH

England

Tel +44 81 783 1152

Fax +44 81 783 1223



Reader Service No.65

Custom DNA

Purified and Shipped in 48 hours,
Worldwide.

100 ug, \$10.00 a base for the first 15 bases,
\$7.50 for each additional base.

Delivered.

Research Genetics

1-800-533-4363

In the United Kingdom 0-800-89-1393

In Canada 1-800-533-4363

FAX 205-536-9016

Reader Service No.32

Synthetic Oligonucleotides

Custom made probes and primers,
synthesised, purified and shipped in 3 days

£3.50 per base

(All inclusive)

0.2 µM scale (approx. 500 µg)

Modified bases, biotin or digoxigenin labelling,
data base analysis and full design facilities

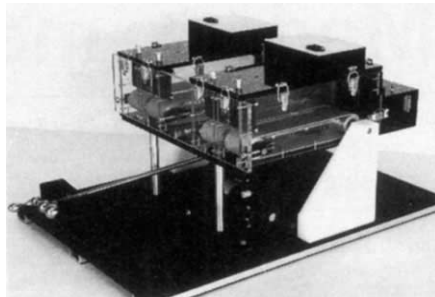
Molecular Medicine Unit,

King's College School of Medicine & Dentistry,
Denmark Hill, London SE5 8RX

Tel. XX-44-(0)71 - 326 3126

FAX XX-44-(0)71 - 733 3877

Reader Service No.56



Four-animal exercise treadmill.

(Reader Service No. 105). The treadmill features four separate airtight animal compartments, each of which is equipped with its own belt and air mixing fan. In order to measure O₂ consumption, air from the compartments is circulated through O₂ and CO₂ sensors. Both the speed and inclination of the treadmill can be adjusted, says Columbus. In addition, the treadmill is fitted with either an electrical or air-puff stimulus to stimulate the animals to exercise.

Counting devices

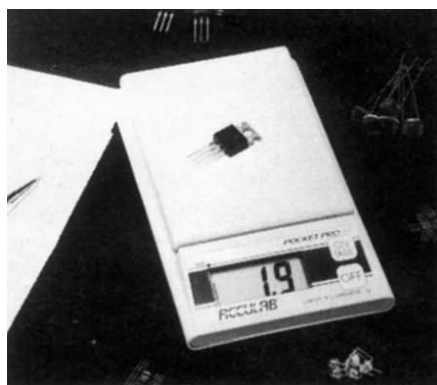
The multidetector 1450 MicroBeta liquid scintillation counter from Pharmacia is designed to cut down on both consumables and radioactive waste: samples are counted directly in microtitre plates and so the need for vials is eliminated (Reader Service No. 106). The six detectors of the 1450 MicroBeta are designed to provide high throughput with beta- or gamma-labelled samples. The MicroBeta has a sample capacity of up to 1,536 for 96-well microtitre plates and 384 for 24-well plates. The counter, which provides the user with an automatic d.p.m. program, chemiluminescence correction and built-in data management software, can be interfaced with automatic sample preparation systems.

An upgraded version of the Protos colony counter from Ai Cambridge was launched earlier this month with a host of additional functions designed to make life easier for the microbiologist (Reader Service No. 107). Measurements can be made on a variety of colonies, including bacteria, cells and plaques, and on media such as opaque and transparent agars, membrane filters and agarose gels. Protos can count objects of any

shape, says Ai Cambridge, provided that there is a detectable difference in contrast between the sample and the background. In addition, users can now vary the colour coding of the displayed image to assist in identification and detection. Algorithms for debris counting have been incorporated and user-defined frames can be set up to scan areas not overgrown by spreading colonies, with the software allowing extrapolation to the full frame. A macro facility is available for researchers carrying out repetitive tasks.

Weight watchers

Are you looking for a balance, but either space is at a premium or portability is desirable. Lux Scientific may have the answer. The company is offering Pocket Pro pocket-sized balances, which weigh in g, oz, dwt and

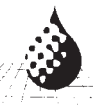


Lux's lightweight balances.

ozt, and are available in 80-g, 150-g and 250-g capacities (Reader Service No. 108). These battery-operated digital balances are graduated in 0.1-g increments and have a precision of ±0.1 g, says Lux. Each Pocket Pro balance is supplied with a calibration weight and carrying case.

All-round access and visibility are the key design features of the RC Series of analytical balances from Sartorius (Reader Service No. 109). Access to the weighing chamber is achieved by turning the movable draft shield and adjusting the width of the opening to suit. With a single keystroke, the door will close and the opening position will be held in the instrument's memory for future applications. The door can be opened to an extra-wide angle of 170° for robotic weighing applications. The RC Series includes balances with weighing ranges from 0–60 g and from 0–250 g, and a readability range of 0.01–0.1 mg. Response times are less than 10 seconds, says Sartorius. An automatic calibration function compensates for any drift that may occur as a result of adverse ambient conditions. The RC Series can be connected to computers for remote control operation. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.



**Severn
Biotech Ltd.**

TM

Custom DNA Synthesis
Probes • Primers • Oligo's

*For a fast efficient
Cost effective service*

BUY SEVERN BIOTECH

Tel: 0562 825286 Fax: 0562 825284

Unit 23, Hoobrook Enterprise Centre,
Worcester Road, Kidderminster DY10 1HY

Reader Service No.7